VOLUME 39 JANUARY 1961 NUMBER 1

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Canadian Journal of Biochemistry and Physiology

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FURTHER OBSERVATIONS ON METABOLIC ALTERATIONS IN THE HYPOTHERMIC RAT[1,2]

JOHN R. BEATON

Abstract

Further observations on metabolic alterations in fasted rats cooled under ice to rectal temperatures approximating 15° C are reported. In the hypothermic rats, metabolism of injected lactic acid does not appear to be impaired. There is however: increased concentration of inorganic phosphorus in blood but not in liver; increased concentration of glutathione in liver but not in blood; increased plasma chloride concentration; decreased red cell potassium concentration; increased red cell water content; decreased plasma water content. Hypothermia, under these conditions, did not alter concentrations of liver acid-extractable glycogen, red cell sodium, plasma sodium, plasma potassium, nor serum calcium. Administration of urea in saline prior to cooling elevated plasma sodium and potassium concentrations in hypothermic rats. These observations are discussed in relation to previously reported effects of hypothermia on carbohydrate metabolite levels.

Introduction

The literature on hypothermia is profuse but has been reviewed recently (1, 2). In a recent communication from this laboratory (3), it was reported that prior administration of urea or of acetazoleamide permits successful resuscitation and survival, in a normal condition, of rats cooled to rectal temperatures of 0–3° C. Subsequently (4), we investigated metabolic alterations occurring in hypothermic rats and the effect of urea treatment on these in an attempt to ascertain the mechanisms through which urea exerts its beneficial action in hypothermia. It was found that prior treatment with urea prevents the significant decrease in blood glucose and increase in blood lactic acid occurring in fasted rats cooled to 15° C rectal temperature; urea was without effect on blood pH, packed cell volume, inorganic phosphorus, acid-soluble phosphorus, and amino nitrogen in hypothermic rats.

The present studies were undertaken to confirm, extend, and add to our

[1]Manuscript received August 4, 1960.
Contribution from Defence Research Medical Laboratories, Toronto, Ontario.
[2]DRML Report No. 109-4, PCC No. D50-93-10-72.

observations on metabolic alterations in the hypothermic rat. The effect of pretreatment with urea on certain of these alterations has been investigated.

Materials and Methods

Adult, female rats of the Wistar strain, maintained on fox chow and drinking water ad libitum, were used. Food was removed 18 hours prior to experimental treatment to eliminate its variable effects upon the metabolites under study. Induction of hypothermia and withdrawal of blood samples were carried out as in the previous study (4). Protein-free filtrates were rapidly prepared from heparinized blood obtained from the exposed heart (right ventricle) and were analyzed as follows: glucose, Folin and Wu (5); lactic acid, Barker and Summerson (6); inorganic phosphorus, Fiske and Subbarow (7); glutathione, Grunert and Phillips (8). Blood electrolytes and water were measured as follows: sodium and potassium by standard flame photometric techniques using an EEL flame photometer; chloride by the modified method of Schales and Schales (9); serum calcium by the flame photometric method of Powell (10); water by drying samples weighing approximately 1.0 g to a constant weight in a temperature-controlled oven at 105° C. In the case of electrolyte and water determinations, blood was withdrawn anaerobically and centrifuged rapidly, and the plasma and red cells separated. Preliminary tests showed that use of heparin as an anticoagulant did not interfere with these determinations. In certain experiments, liver homogenates were prepared in the cold using homogenizing tubes and teflon pestles. These homogenates were analyzed for acid-extractable glycogen (11), inorganic phosphorus (7), and glutathione (8).

In studies on liver acid-extractable glycogen, blood electrolytes, and blood water, the effect of urea was examined. As in the previous study (4), rats were injected intraperitoneally with 3.0 ml of 0.85% aqueous sodium chloride containing urea at a level to provide 120 mg urea/100 g body weight. Control animals received an intraperitoneal injection of 3.0 ml 0.85% aqueous sodium chloride. A period of 8 minutes was allowed to elapse between injection and initiation of cooling to permit absorption of sufficient urea to yield a significantly elevated level of blood urea (4).

Results are expressed as mean values ± standard error for each group of animals. The significance of differences between means was calculated by application of Student's "*t*" test. Although significance of difference was tested in every case, probability values are shown only in those cases where $P < 0.05$.

Results

Lactic Acid Injection

Following the earlier observation that blood lactic acid is elevated in the fasted, hypothermic rat, 32 rats were divided into four groups of 8 each: one group served as an initial control; one group was cooled under ice for 32 minutes; the remaining two groups were injected intraperitoneally with an aqueous solution of 15% lactic acid, adjusted to pH 7.0 with sodium hydroxide,

in a volume to provide 150 mg lactic acid/100 g body weight. Of the latter two groups, one was sacrificed 40 minutes after injection without cooling; 8 minutes after lactate injection, the other was cooled under ice for 32 minutes and then sacrificed. Blood levels of glucose and lactic acid were determined. As shown in Table I, the previously reported decrease in blood glucose and

TABLE I

Average blood lactic acid and glucose levels following intraperitoneal injection of lactic acid (150 mg/100 g body weight) (eight rats/group)

Group	(1) Control	(2) Control, lactate-injected	(3) Hypothermic	(4) Hypothermic lactate-injected	Significance of difference, *P*
Body weight, g	241 ± 5.4	216 ± 5.4	237 ± 5.4	224 ± 4.6	—
Rectal temp., °C	—	—	16 ± 0.59	16 ± 0.49	—
Blood glucose, mg%	104 ± 4.3	191 ± 9.3	86 ± 1.1	136 ± 8.2	(1) vs. (2) <.001 (1) vs. (3) <.01 (3) vs. (4) <.001 (2) vs. (4) <.001
Blood lactic acid, mg%	42 ± 3.9	138 ± 3.9	60 ± 3.6	124 ± 4.1	(1) vs. (2) <.001 (1) vs. (3) <.01 (3) vs. (4) <.001 (2) vs. (4) <.05

increase in blood lactic acid in untreated, fasted, hypothermic rats (4) were confirmed. Forty minutes after lactic acid injection, uncooled and hypothermic rats exhibited significantly elevated blood levels of glucose and lactic acid in comparison with animals not given lactic acid. However, the mean elevation in blood lactic acid after injection was markedly less in hypothermic rats (64 mg%) than in control animals (96 mg%).

Blood and Liver Concentrations of Inorganic Phosphorus and Glutathione

Determinations of inorganic phosphorus and glutathione levels were made on blood and liver tissue of 10 uncooled and 10 hypothermic rats; the results are shown in Table II. The increase in blood inorganic phosphorus (4) consequent upon hypothermia in fasted rats was confirmed. A statistically insignificant

TABLE II

Average blood and liver concentrations of inorganic phosphorus and glutathione in control and hypothermic rats (eight rats/group)

Group	Control	Hypothermic	Significance of difference, *P*
Body weight, g	222 ± 5.7	222 ± 6.0	—
Rectal temp., °C	—	14 ± 0.33	—
Blood inorganic phosphorus, mg%	5.9 ± 0.31	20.8 ± 0.37	<.001
Liver inorganic phosphorus, mg%*	40 ± 2.2	55 ± 5.4	—
Blood glutathione, mg%	31 ± 1.8	34 ± 0.8	—
Liver glutathione, mg%*	100 ± 3.0	203 ± 11.4	<.001

*Wet weight basis.

increase in liver inorganic phosphorus accompanied the increase in blood level. Hypothermia resulted in a significant increase in the concentration of glutathione in liver but not in blood.

Liver Acid-extractable Glycogen Concentration

Acid-extractable glycogen concentrations were determined in liver tissue of 10 control rats, 10 cooled rats, and 10 urea-treated, cooled rats. Care was taken to minimize activity in these animals since it has been shown that this fraction of liver glycogen is labile and highly susceptible to activity, as well as to fasting (12). As shown in Table III, depletion of this glycogen fraction was severe in

TABLE III

Liver acid-extractable glycogen levels in control, hypothermic, and urea-treated hypothermic rats (10 rats/group)

Group	Body weight, g	Rectal temp., °C	Liver acid-extractable glycogen, %*
Control	218±2.6	—	0.15±0.010
Hypothermic	218±2.7	13±0.7	0.12±0.003
Urea-treated, hypothermic	218±2.6	12±0.7	0.12±0.006

*Wet weight basis.

all groups, probably as a result of the fasting. Hypothermia under these conditions was without effect upon acid-extractable glycogen concentration of the liver, whether or not urea was administered prior to cooling.

Blood Electrolyte and Water Concentrations

Four groups of 10 rats each were used: a saline-injected control group; a saline-injected group cooled for 27 minutes; a urea-treated group sacrificed 35 minutes after injection; a urea-treated group cooled for 27 minutes. Sodium, potassium, and water were determined in both red cells and plasma. Chloride was determined in plasma only. The results of this experiment are shown in Table IV. Hypothermia caused decreased concentrations of red cell potassium and plasma water and increased concentrations of plasma chloride and red cell water. Administration of urea to non-cooled animals increased red cell water and plasma sodium levels. When administered prior to cooling, urea increased plasma potassium and sodium levels in hypothermic rats. Although urea treatment reduced the elevation in plasma chloride level due to cooling, this elevation was still significantly higher than in non-cooled animals. The observation that concentration of potassium in the red cell is decreased (16.8%) in hypothermic rats can be explained only in part by the increase in the water content of the cell (7.7%). Similarly, the increased plasma chloride concentration (10.3%) is only partially explained by the decrease in plasma water (2.4%).

Serum Calcium Levels

Due to the relatively large quantity of serum required (2 ml) for this estima-

TABLE IV

Blood electrolyte and water levels in control, hypothermic, urea-treated, and urea-treated hypothermic rats (10 rats/group)

Group	(1) Control	(2) Hypothermic	(3) Urea-treated	(4) Urea-treated hypothermic	Significance of difference, *P*
Body weight, g	206 ± 8	250 ± 5	216 ± 7	259 ± 4	—
Rectal temp., °C	—	14 ± 0.6	—	14 ± 0.6	—
Red cell sodium, meq/l.	18 ± 1.9	22 ± 1.2	17 ± 1.6	24 ± 1.9	—
Plasma sodium, meq/l.	158 ± 3.1	159 ± 2.5	177 ± 2.8	197 ± 7.3	(1) vs. (4) < .001 (1) vs. (3) < .001 (2) vs. (4) < .001
Red cell potassium, meq/l.	101 ± 1.6	83 ± 1.3	102 ± 0.9	85 ± 2.5	(1) vs. (2) < .001 (1) vs. (4) < .001
Plasma potassium, meq/l.	6.4 ± 0.16	6.1 ± 0.38	6.2 ± 0.22	8.2 ± 0.66	(1) vs. (4) < .02 (2) vs. (4) < .02 (3) vs. (4) < .02
Plasma chloride, mg%	379 ± 3.50	418 ± 0.32	386 ± 3.10	398 ± 1.58	(1) vs. (2) < .001 (1) vs. (4) < .001 (2) vs. (4) < .001
Red cell water, %	64.1 ± 0.79	69.0 ± 0.60	68.6 ± 0.66	67.8 ± 1.10	(1) vs. (2) < .001 (1) vs. (3) < .001 (1) vs. (4) < .02
Plasma water, %	91.0 ± 0.69	88.8 ± 0.41	91.9 ± 0.50	89.1 ± 0.25	(1) vs. (2) < .02 (1) vs. (4) < .02 (3) vs. (4) < .02

tion, it was not possible to carry out serum calcium determinations in conjunction with measurement of other blood electrolyte levels. Calcium determinations were made on serum from 14 saline-injected control, 9 saline-injected cooled, and 10 urea-injected cooled animals. From the results shown in Table V, it is apparent that hypothermia was without effect upon serum calcium levels whether or not urea was administered prior to cooling.

TABLE V

Serum calcium concentrations in control, hypothermic, and urea-treated hypothermic rats

Group	Number rats	Body weight, g	Rectal temperature, °C	Serum calcium, meq/l.
Control	14	201 ± 1.9	—	5.6 ± 0.21
Hypothermic	9	207 ± 2.7	15 ± 0.7	6.1 ± 0.47
Urea-treated hypothermic	10	200 ± 1.4	13 ± 0.6	5.6 ± 0.28

Discussion

The results shown in Table I demonstrate that injected lactic acid is cleared from the blood at least as rapidly in hypothermic rats as in uncooled rats. Preliminary studies showed that the maximum blood level of lactic acid occurs in 10 minutes or less following lactate injection; since cooling was not initiated until 8 minutes after lactate injection, it is unlikely that the lower blood level of lactic acid in the lactic-injected, hypothermic rats is a consequence of reduced absorption due to cooling. It is concluded, therefore, that the elevated blood lactic acid level of fasted, hypothermic rats is the result of increased lactic acid production rather than decreased utilization.

The results shown in Table II suggest that the increase in blood inorganic phosphorus of fasted, hypothermic rats is not inorganic phosphorus mobilized from the liver but represents breakdown of organic phosphorus compounds. Kaplan (13) states that an accumulation of inorganic phosphorus accelerates metabolism of carbohydrate and is unfavorable to glycogen synthesis. The findings shown in Table III provide evidence that alterations in carbohydrate metabolites in fasted, hypothermic rats do not result from alterations in liver content of glycogen.

Glutathione is believed to be directly involved in carbohydrate metabolism (14) probably as the prosthetic group of 3-phosphoglyceraldehyde dehydrogenase (15). The increased liver concentration of glutathione (Table II) could be indicative of a decrease in 3-phosphoglyceraldehyde concentration or of some alteration in oxidation–reduction reactions dependent upon a free sulphydryl group.

Neither anaesthesia nor artificial ventilation was administered in the cooling phase of our studies. When artificial ventilation is used during cooling, the resultant hyperventilation per se causes a decrease in serum potassium concen-

tration (16); and under the influence of anaesthesia, potassium enters cells (17). It has been considered that potassium follows the cycle of carbohydrate metabolism, so that potassium is increased when blood lactic acid is elevated as in muscular exercise, increased metabolism, and anoxia (17). Elevated cardiac potassium has been observed in hypothermic dogs (18). Other workers (19) have attributed increased serum potassium concentrations in cooled rats to release of potassium by the breakdown of hepatic glycogen. This is unlikely in our fasted, hypothermic animals (Table III). The decrease in red cell potassium observed in our hypothermic rats (Table IV) may reflect an alteration in metabolic activity of these cells or simply an entrance of potassium into cells other than erythrocytes in response to the marked increase in lactic acid concentration. Elevated plasma potassium levels in urea-treated, hypothermic rats may be related to the effect of urea in preventing decreased blood glucose and increased blood lactic acid in such animals (4).

Spurr and Barlow (20) have recently reported increased plasma chloride in the hypothermic dog, which is in agreement with the present observation in hypothermic rats. It is generally observed that chloride and water move conjointly, for example to plasma when blood is oxygenated; further, a reduction in blood pH is usually accompanied by an increase in chloride concentration. In our hypothermic rats, plasma water content decreased and plasma chloride increased and such animals exhibit a lowered blood pH (4). When urea was administered prior to cooling, the increase in plasma chloride was accompanied also by an increase in plasma sodium concentration.

An increased concentration of serum calcium in hypothermia has been reported (19, 21) but was not observed under our conditions.

The apparent movement of water from plasma to red cells in hypothermic rats may reflect an alteration in erythrocyte metabolism. Rixon and Stevenson (22) using rat diaphragm in vitro demonstrated that inhibition of respiratory metabolism by low temperatures resulted in a net uptake of water by cells. In our animals, the increased water content of erythrocytes, and consequent increase in size, probably explains the increased blood packed cell volumes observed (4) since unpublished studies in our laboratory have failed to show an increase in the red blood cell count of such animals.

In summary, the findings reported here have confirmed and extended our previous observations (4). It is emphasized that a specific set of experimental conditions has been employed: fasted rats, no anaesthesia, and no artificial ventilation. At least some of our observations are consistent with an hypothesis that in hypothermic rats a relative anoxia exists, that is, available oxygen is insufficient to meet the reduced metabolic activity as a consequence of the reduced respiratory activity and circulation. In general, administration of urea to non-cooled animals brings about the same alterations in blood electrolyte and water concentrations as does hypothermia, but urea and hypothermia conjointly do not have an additive effect.

Acknowledgment

The author is indebted to Mr. T. Orme for his valuable technical assistance throughout the course of these studies.

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INSULIN SENSITIZATION TO THE ACTION OF *k*-STROPHANTHIN[1]

V. W. ADAMKIEWICZ

Abstract

Insulin-Zn (40 units/kg, s.c.) prolongs two- to four-fold the cardiac action of the glycoside *k*-strophanthin (10–20 mg/kg, s.c. or i.p.) in the rat (100–200 g) as reflected by the duration and intensity of the heart rate deceleration (E.C.G.). Insulin also increases six times the mortality in rats overdosed with *k*-strophanthin (30 mg/kg i.p.), but simultaneously prolongs the duration of survival. On the other hand, insulin appears not to affect the cardiac action of the aglycon strophanthidin.

Insulin potentiates (1, 5), while diabetes inhibits (2, 3), in rats, the biological activity of the glucan dextran. The last effect is reminiscent of the observations of Travis, Keyl, and Dragstedt (4) that diabetes inhibits, in the dog, the biological activity of the cardioactive glycoside *k*-strophanthin. Dextran and *k*-strophanthin possess in their structures a terminal molecule of glucose. On the basis of these analogies, we investigated the influence of insulin on the biological activity of *k*-strophanthin.

Methods

We used male Sprague-Dawley rats, 100–140 g in body weight, maintained on a standard diet of Purina cubes and water. The ambient temperature of the animal house and of the laboratory during the experiments was $26 \pm 3°$ C.

The doses of *k*-strophanthin (K & K Laboratories) were in experiment 1: 20 mg/kg, intraperitoneally (i.p.); in experiment 2:15 mg/kg, subcutaneously (s.c.) on the back; and in experiment 4: 30 mg/kg, i.p. They were administered immediately after preparation, in a volume of 1 ml of saline per rat. Strophanthidin (Sandoz), the cardioactive aglycon of *k*-strophanthin, was administered in experiment 3 at the dose of 15 mg/kg, s.c., in 5 ml of saline per rat.

Insulin-Zn ("Insulin-Toronto," Connaught) was injected throughout in the dose of 4 units per rat, s.c. on the back, alone or simultaneously with other drugs.

The number of heart beats per minute was measured with an electrocardiograph (E.C.G., Sanborn). Without using anesthesia, three safety pins were attached to the skin of rats: on the right and left sides of the chest, and across the base of the tail. The first two pins were connected to the "right" and "left" arm leads of the E.C.G., and the third was grounded. The rat was held in one hand and its electrocardiogram was recorded. Each R wave was taken to represent one heart beat (Fig. 1). The number of R waves per minute were

[1]Manuscript received June 29, 1960.
Contribution from the Department of Physiology, Faculty of Medicine, University of Montreal, Montreal, Que. This study was supported by research grant No. MA 640 from the National Research Council of Canada and research grant No. A-2260 from the U.S. Department of Health, Education and Welfare.

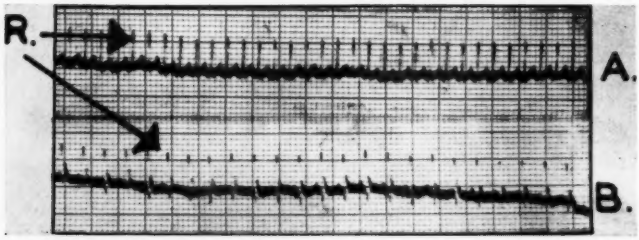

FIG. 1. E.C.G. in the rat. R: R waves; A: heart rate of normal rat; B: decelerated rate of rat treated with *k*-strophanthin.

obtained for each rat. They were then used to calculate the mean heart rate per minute, at the time of measurement, in each experimental group. The chronology of the procedure was as follows. The safety pins were placed in all the animals. Two normal E.C.G.'s were read at -1 and at 0 hours. Then the animals were injected with the drugs. E.C.G.'s were then obtained again at $\frac{1}{2}$, 1, 2, 3, 4, 5, and 6 hours.

The influence of insulin on the lethal effect of *k*-strophanthin was studied in experiment 4 by counting the rats which were dead within 24 hours after the injection of the drugs. The time between the injection and death was noted (duration of survival). In experiments 1, 2, and 3 the results were analyzed statistically by Student's "*t*" test, using paired values at the various times. In experiment 4 the mortality rate was evaluated by the Chi-square test.

Results

1. Sensitization by Insulin of the Cardiac Action of k-Strophanthin Injected i.p.

Four groups of 15 rats were used. Group I rats were untreated, group II received insulin, group III, *k*-strophanthin, and group IV, insulin and *k*-strophanthin at the same dose as the previous groups.

The mean heart rate of the untreated rats was 515 ± 15 S.E. beats per minute and remained approximately at this level for 8 hours. The insulin-treated animals had a mean heart rate of 495 ± 14 before as well as after the injection. The rate remained at this level during 8 hours. The mean heart rate of the *k*-strophanthin-treated rats was 465 ± 16 before the injection. Half an hour after injection, it decreased rapidly to its lowest point of 390 ± 13 beats per minute. Then it rose quickly and had returned to normal after 2.5 hours (0 hour/1 hour, $P < 0.01$; 0 hour/3, 4, 6, 8 hours, $P > 0.9$). Finally the heart rate of rats injected with *k*-strophanthin plus insulin which was 485 ± 11 before the injection slowly decreased after the injection reaching its lowest point of 410 ± 15 beats at the fourth hour. Then it slowly rose, returning to normal at the eighth hour (0 hour/1, 2, 3, 4, 6 hours, $P < 0.05$; 0 hour/8 hour, $P > 0.9$) (Fig. 2).

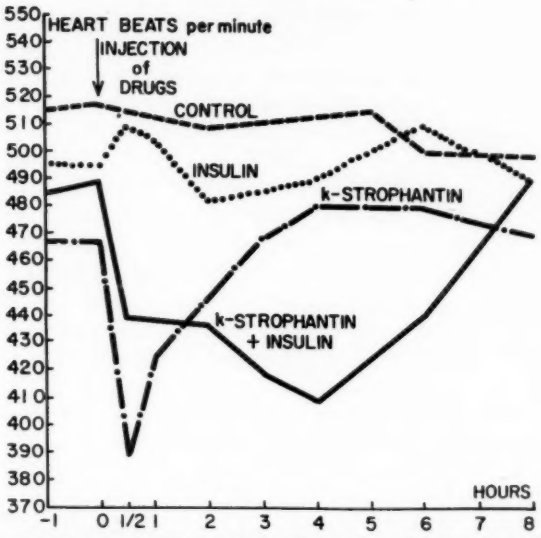

FIG. 2. Sensitization by insulin to the cardiac action of *k*-strophanthin injected i.p. Abscissa: time. Ordinate: heart beats per minute. Arrow marks time of injection of drugs (0 hour). The heart rate of control and insulin-treated rats remains approximately stable. *k*-Strophanthin induces a rapid, transient deceleration of rate which lasts 2 hours. *k*-Strophanthin plus insulin induce a more gradual, but very protracted deceleration which lasts to the eighth hour.

2. Sensitization by Insulin of the Cardiac Action of k-Strophanthin Injected s.c.

Four groups of 15 rats were treated as previously, except that the injections were made subcutaneously.

The heart rate of the controls was 490 ± 15 and showed a tendency to decrease during the 6 hours of the experiment. The insulin-treated animals had a rate of 470 ± 18 before the injection. It maintained itself at approximately this level after the injection showing, however, a tendency to fall as in the controls. The heart rate of the *k*-strophanthin-treated rats, which was 450 ± 13 before the injection, rapidly decreased after injection to its lowest point of 370 ± 21 at the first hour. Then, it rapidly returned to normal in the second hour, and maintained itself at the normal level until the end of the experiment (0 hour/1 hour, $P < 0.01$; 0 hour/2, 3, 4, 5, 6 hour, $P > 0.9$). Finally, the rats injected simultaneously with *k*-strophanthin and insulin, which, before the injection, had a rate of 480 ± 10, showed a slow decrease after injection, reaching the lowest point of 400 ± 20 at the third hour. The rate returned to normal at the fifth hour (0 hour/2, 3, 4 hour, $P < 0.01$; 0 hour/5, 6 hour, $P > 0.9$).

3. Interaction between Insulin and the Cardioactive Aglycon Strophanthidin

Ten rats of one group were injected with the aglycon strophanthidin, and 10 rats of another with the same dose of the aglycon plus insulin.

Before the injections, the heart rate of both groups was about 450 ± 10. Within the first hour after injection it rapidly decreased to the lowest point of 385 ± 18 beats. Then it rose slowly until the sixth hour. Despite the difference in treatment, the two groups of animals did not differ in the pattern of their heart rate (Fig. 3).

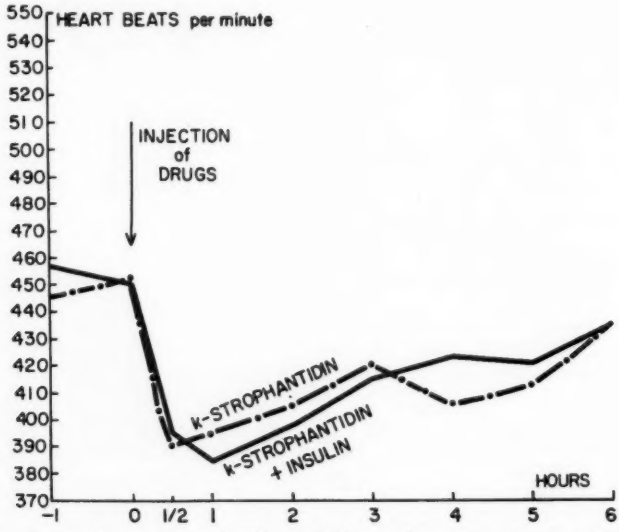

FIG. 3. Interaction between insulin and the cardioactive aglycon strophanthidin. Insulin appears not to affect the action of the aglycon on the heart rate.

4. Potentiation by Insulin of the Lethal Effect of k-Strophanthin

One hundred rats were injected i.p. with a slight overdose of *k*-strophanthin. A second group of 100 rats received the same dose of *k*-strophanthin plus insulin. Fifty rats of a third group received insulin alone.

Four rats died in the first group. The average duration of survival was 45 ± 6 minutes. In the second group, 24 rats died. The average duration of survival was 394 ± 43 minutes (6 hours, 34 minutes, χ^2 group I/group II$\simeq 15$, $P < 0.001$). All the rats of the third group survived. Most of the rats treated with insulin plus *k*-strophanthin underwent asymmetric convulsions sometime during the first 12 hours after injection. The bouts of convulsions lasted 2 to 6 hours.

Discussion

The normal mean heart rate of male Sprague-Dawley rats, 90–200 g in body weight, is 481 ± 15 S.E. heart beats per minute. Although the rate varies from rat to rat and from day to day, the mean rate in any one group remains constant for any one period of 4 to 8 hours.

The glycoside *k*-strophanthin and the aglycon strophanthidin, injected i.p. or s.c., at doses of 10 to 20 mg/kg, rapidly decelerate the heart. Within the first hour after administration, the rate falls to its lowest value of about 380 ± 21 beats per minute; a deceleration of 21%. In the case of the glycoside, the rate returns to normal within 2 hours. But, in the case of the aglycon, the return is slower.

In insulin plus *k*-strophanthin-treated animals, the deceleration sets in also within the first hour, but it reaches the lowest point more gradually, between the third and fifth hour. The complete return to the normal rate is delayed to between the fourth and eighth hour. Therefore, insulin prolongs the action of *k*-strophanthin two- to four-fold.

On the other hand, insulin appears not to affect the action of the aglycon strophanthidin. The aglycon differs from the glycoside by the absence of the sugar side-chain, including a terminal glucose.

Rats overdosed with *k*-strophanthin die within the first hour after injection as a rule. Thus, the time of death corresponds to the time of the greatest slowing of the heart. Insulin increases six times the mortality rate of rats slightly overdosed with the glycoside and delays about six times the time of death. This increased mortality, and the delayed time of death, are a reflection of the delay in the slowing of the heart and of the prolonged action of *k*-strophanthin on the heart induced by insulin.

The rats overdosed with *k*-strophanthin and treated with insulin often undergo convulsions which do not necessarily result in death. One explanation of this phenomenon may be that under the influence of insulin the glycoside penetrates and affects tissues which normally are resistant to it, such as nerves or muscles. But the secondary effects of the prolonged depression of the heart can not be excluded as the cause of convulsions.

Acknowledgment

The author expresses his thanks to Dr. J. Saint-Pierre, Director of the Department of Statistics, University of Montreal, for the statistical evaluation of the data.

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THE ENTEROLIPOMICRON, A SECRETION?[1]

C. B. WELD

Abstract

Enterolipomicrons, small particles visible in dark field preparations, appear in Thiry loops of duodenum or jejunum on physiological stimulation. Usually, debris consists of desquamating, disintegrating mucosal cells; mucous material is obtained at the same time. The particles differ from the debris in composition and enzyme activity, and their appearance and their composition suggest a cytoplasmic origin. In electron microscope preparations of resting mucosa, similar particles can be seen in large numbers in the cells whereas in vigorously stimulated preparations they are scarce. It is concluded that the centrifugal fraction normally called enterolipomicron consists of a mixture of secretory granules actually secreted and of cytoplasmic microsomal material derived from disintegrating cells. The fraction has considerable enzymatic activity.

Introduction

Singer, Sporn, and Necheles (1) gave the name enterolipomicron to chylomicron-like particles obtained from jejunum Thiry loops in dogs. They reported that the particles were obtained both from fasting animals and from animals exhibiting alimentary lipemia, and they suggested that the particles might be concerned in some way with the excretion of lipids from the body. It was of interest to study further the composition and properties of these particles in order to ascertain their physiological significance.

Methods

Thiry loops were prepared, 8–12 in. long, in dogs, (*a*) of the duodenum below the pancreatic duct to the upper portion of jejunum, (*b*) of jejunum alone, the upper end being a few inches below the ligament of Treitz, and (*c*) of the lower ileum. Very little material and few particles were obtained from any of these resting loops, but they were obtained in abundance from the upper loops when stimulated by distention in the following way.

A catheter was led through a small balloon and passed into the loop. The balloon acted as a stopper or plug. Sufficient warm saline (.15 *M* NaCl) was then injected through the catheter to distend the loop to a pressure of 4–8 in. of saline. The quantity of saline required varied from animal to animal; in most cases it was between 10–12 ml unless pilocarpine was given, when 2–5 ml sufficed. Within a very few minutes rhythmical contractions were evident and mucous–cellular debris and a large number of the enterolipomicrons appeared in the saline. From time to time the saline was withdrawn and replaced by fresh solution. For the most part in these experiments the period of stimulation was 30 minutes and the collections were made at 10-minute intervals. The

[1]Manuscript received May 31, 1960.
Contribution from the Department of Physiology, Dalhousie University, Halifax, N.S. This work was supported by a grant from the National Research Council of Canada.

saline material was then centrifuged in an International Clinical Centrifuge for 30 minutes and the "debris" removed. The particles still remaining in suspension were considered to be the enterolipomicrons. These were then washed by centrifuging at 18,000–20,000 *g* for 1 hour and resuspending in saline, 1–3 times, depending on the experiment. The "debris" was suspended in saline and homogenized.

Bacteria were present, and it was not found possible to separate them completely from the enterolipomicrons by differential centrifugation. However, by immediately chilling the specimens in ice, doing the centrifuging in the cold room (4° C), and keeping the specimens well refrigerated, it was felt that the numbers were kept sufficiently under control. Using these precautions, only a very few bacteria were seen in stained smears.

Total lipid determinations of the fractions were done by a modified Sperry–Folch method (2) extracting the lipid with chloroform–methanol, shaking the mixture with water, and weighing the residue after it was dried under radiant heat. Nitrogen was done by micro-Kjeldahl and titration; protein was calculated by the factor 6.25. The results reported give the proportions of lipid to protein. Lipid phosphorus was determined by the method described by King (3), and converted to lecithin values by the factor 25. Total cholesterol was done by the modified Sackett method described by King (4). Both the phosphorus and cholesterol determinations were made on the dried lipid obtained by the Sperry–Folch extraction. The RNA and DNA fractions of nucleic acid were determined by the method described by Colowick and Kaplan (5).

The enzymatic activity of the fractions was determined as follows. The sucrase activity was determined by incubating the particles with an equal volume 10% sucrose and determining the yield of reducing sugar by the Folin and Malmros (6) method. The activity is expressed in terms of the yield of reducing sugar in g per mg nitrogen. The alkaline phosphatase was done by the Bodansky method (7) and the activity expressed in terms of Bodansky units per mg nitrogen. The cytochrome oxidase activity was determined qualitatively by the microspectrophotometric method of Cooperstein and Lazarow (8). The succinic dehydrogenase activity was determined qualitatively, using a succinic acid substrate and methylene blue as the indicator. The peptidase activity of the material was tested first unsuccessfully using gelatine as a substrate and then successfully using phenol red – fibrin as a substrate. Drops of the material to be tested, at pH 8, were placed in a Petri dish along with suitable control drops of saline and of known dilutions of trypsin. A few grains of the solid phenol red – fibrin complex were then added to each drop and the whole set incubated an hour at 38° C. The liberation of phenol red into the drop constituted a positive reaction. The catalase activity was noted qualitatively by adding the material to 1% H_2O_2, the appearance of bubbles indicating a positive reaction. Quantitative determinations were done by the method described by Chantrenne (9). These are reported on a comparative basis. The esterase activity was determined by the Knaffl-Lenz procedure,

referred to by Stotz (10). Ethyl butyrate was used as substrate and the esterase activity is expressed as ml of 0.01 *N* NaOH per mg of nitrogen required to neutralize the liberated butyric acid.

Results

Visually, under dark field illumination, the particles were dancing bright particles, as described by Singer, Sporn, and Necheles (1), and ranged in size from the barely visible up to about one micron. It is difficult to estimate either the size or the shape of such minute particles but the impression is received that many of the particles are irregular in form and not spherical whereas chylomicrons of blood usually seem to be spherical.

Preparations, air-dried, fixed with alcohol, and stained with methyl green – pyronin B were not readily resolvable under oil immersion at 1000× but they did take the pyronin stain, suggesting a high ribonucleic acid content. Their appearance in electron microscope preparations is described later.

The debris fraction consisted of cellular material in all stages of disintegration, and of mucus. The presence of the cellular material is to be expected in view of the report of Leblond and Stevens (11) on the constant renewal of the intestinal epithelium (rat).

The composition and enzymatic activity of the various particles and fractions is shown in Tables I and II.

TABLE I

Composition of particles and debris

	Chylomicron	Enterolipomicron (n = 20)	Debris (n = 20)
Protein	10%	56.0%	64.4%
Lipid	90%	44.0% ±2.3*	35.6 ±2.8*
Lecithin, mg/mg lipid	.05	.19 ± .017*	.15 ± .012*
Cholesterol, mg/mg lipid	.02	.08 ± .008*	.06 ± .005*
RNA (n = 8), mg/mg N_2		.024 ± .001*	.018 ± .001*
DNA (n = 8)		.018 ± .004*	.057 ± .001*
Mean of differences, enterolipomicron − debris,			
Lipid	8.4,	$P < .01$.	
Lecithin	.04,	$P < .05$.	
Cholesterol	.02,	$P < .01$.	
Mean of ratios, enterolipomicrons/debris,			
RNA	1.71,	$P < .01$.	
DNA	0.50,	$P < .05$.	

*Standard error of mean.

Composition of the Particles

Comparing the enterolipomicron particles with the chylomicron (12), the following differences are noted.

(1) The density of the intestinal particles is much greater; they sediment readily when centrifuged in .16 *M* NaCl solutions whereas the lower density chylomicrons rise to the surface.

TABLE II

Enzyme activity of particles and debris

Activity/mg N_2	Enterolipomicron	Debris	Particles from debris
Alkaline phosphatase (n = 16)	4.0 ±.2*	.7 ±.1*	2.7±.3*
Sucrase (n = 8)	4.3 ±.9*	.9 ±.2*	
Esterase (n = 18)	.023±.002*	.017±.003*	
Catalase (n = 7)	1.0	5.8 ±.6*	5.4±.5*
Cytochrome oxidase	0	+	
Succinic dehydrogenase	0	+	
Peptidase	+	0	

*Standard error of the mean.

(2) The particles have a much lower lipid content than do chylomicrons.

(3) The enzymatic activity of the particles is considerable.

(4) The suspension of the intestinal particles is not cleared by postheparin clearing factor. This was tried on many occasions. Postheparin clearing factor serum, proven to clear suspensions of chylomicrons readily, consistently fails to show any clearing activity when added to the enterolipomicrons.

(5) In confirmation of the results of Singer, Sporn, and Necheles, it has been observed that the enterolipomicrons are obtained whether there is lipemia or not.

From the above evidence it seems abundantly clear that the intestinal particles are not chylomicrons. What then are they? Their composition and activity suggests that they could be of cytoplasmic, possibly microsomal (13, 14) origin. This possibility will now be explored.

It may be seen from Table I that the composition of the enterolipomicron differs from that of the debris. In our earlier experiments the debris was homogenized and washed with saline and a further yield of particles was obtained in the supernatant and added to the enterolipomicrons. It was later realized that the particles derived from the debris differed from the enterolipomicrons, having a lower lipid content and a lower alkaline phosphatase activity. This procedure was discontinued but it is probable that even the minimal manipulations necessary to collect the specimens results in the fragmentation of some of the debris and that our enterolipomicron preparations contain some fraction of debris particles.

This would tend to make our figures for the lipid content of enterolipomicrons somewhat low. In experiments in which stimulation of the loop was continued for an hour, the enterolipomicrons obtained in the latter periods, when there was very little debris collected, contain 50% lipid and it is believed that this figure more truly represents the lipid concentration of the particles obtained by stimulation.

From Table II, it is clear that the enterolipomicron particles contain peptidase, considerable sucrase and alkaline phosphatase, whereas the debris contains but little. On the other hand, the debris, not the enterolipomicrons, shows greater catalase, cytochrome oxidase, and succinic dehydrogenase

activity. As for esterase, though the enterolipomicron figure is larger than the debris figure by a factor of 1.4, the difference is not significant statistically.

These results indicate that the enterolipomicron particles are not merely a simple homogenate of the cellular debris. They could, however, be the microsomal fraction of such a homogenate as obtained by differential centrifugation. That the explanation of their production may not be as simple as this is suggested by the time relationships of their appearance and by histological evidence.

Time of Appearance and Yield of Particles

When the loop is quiescent, few particles are present but when it is distended and becomes active, solid material and many particles promptly appear in the distending fluid. Far more material is produced during this period of activity than can be obtained by simple irrigation of the loop. The particles and debris usually appear together although the yields are not always in the same proportion. In 24 experiments the yields of debris and enterolipomicrons were compared. The yields obtained during a 30-minute stimulation period were determined and in each experiment the ratio of the mg N_2 debris to mg N_2 particle was calculated. The mean of ratios was found to be 7.0 with a standard error of $\pm .9$ and the correlation coefficient (r) between the debris figures and the particle figures was $r = .83$.

Thus it is seen that, although there is a general correlation between the debris yield and particle yield, there are many exceptions. Sometimes there is a good particle yield with very little debris and quite often a large amount of debris with very little enterolipomicron. The occurrence of many exceptions to the general correlation is important because it suggests that the particles are not derived from the debris. Both are produced during intestinal activity, usually together, but not necessarily.

From the point of view of time, the particles appear in greatest numbers in the first few minutes of stimulation and the yield falls to low levels after 15 or 20 minutes. The yield of debris diminishes similarly and usually, but not always, parallels that of the particles.

If the animal is fed an hour before the loop is stimulated by distention, the rhythmical activity of the loop is little changed but the yield of particles is increased by a factor of 2.2 (S.E. $\pm$ .18). This was determined by alternately, at intervals of 3–4 days, feeding and not feeding an animal an hour before the loop was distended and the specimen collected. In these experiments the yield of debris was similarly increased following feeding, the correlation between the debris yield and particle yield being $r = .87$.

The intravenous administration of .25–.50 mg pilocarpine promptly increases the tone and the contractions of the loop as evidenced by the increased displacement of fluid during contraction and by the decreased volume of the loop. Following the injection of these doses of pilocarpine the yield of enterolipomicrons is somewhat increased. Excessive doses of pilocarpine, however, cause

a marked reduction in the yield of enteromicrons, probably because of the extreme tonic contraction of the loop and absence of rhythmic contractions.

The early appearance, on stimulation, of the particles along with cellular and mucinous debris and its lack if there is no stimulation is evidence of a secretory process. However, the method of secretion is not evident and to elucidate this other types of information are required.

Microscopic Examination of Material

Biopsies of mucosa were taken by a biopsy punch from the loops under resting conditions and from the same loops after stimulation. The specimens were immediately frozen by being dropped into isopentane immersed in liquid nitrogen. The dehydration of the fragment of tissue was carried out by Bell's (15) freeze-substitution method, the tissue remaining frozen until dehydration was complete. The specimens were then embedded in paraffin and very thin sections were cut and stained by haemotoxylin and eosin and also stained for mucin. During stimulation, mucus was seen to be expelled from the goblet cells into the lumen where it formed a thin layer on the mucosal surface. After prolonged stimulation there seemed to be fewer goblet cells. No other changes in cell structure were observed in these preparations.

Through the kindness of Dr. Leeson of the Department of Anatomy, we have been enabled to examine electron microscope preparations of our material. The enterolipomicrons, fixed in Dalton's buffer and osmic acid, were seen to have a definite structure. They were small particles often seen in the form of circles, sometimes with a center spot, the largest being less than a micron in diameter and most being a quarter or one-half of a micron. Their appearance is shown in Fig. 1 and is similar to that of the microsome as published in Brachet's monograph (16).

Not all preparations show such consistent appearance. In some a greater variety of particles ranging from amorphous granules to fragments of disintegrating cells is seen. It is unlikely that the method of preparation can do more than crudely separate small fragments of cellular debris from the enterolipomicrons proper. In Fig. 2 a fragment of cell found in the enterolipomicron fraction is shown. In this cell, particles resembling enterolipomicrons are seen and so it becomes clear that some, at least, of the particles identified as enterolipomicrons are derived from disintegrating mucosal cells. The question is whether all are derived in this fashion.

The electron microscope picture of mucous membrane taken with a biopsy punch and immediately fixed in Dalton's buffer and osmic acid also shows similar particles in the cytoplasm of the cells. In addition, the cells reveal beautifully the microvilli that constitute the brush of the cells, the mitochondria, and other structures. These are shown in Figs. 3, 4, and 5. The small vesicular particles that characterize the enterolipomicrons in the lumen are more numerous in specimens of resting mucosa than in specimens from loops that had been subjected to prolonged stimulation. This is shown by comparing

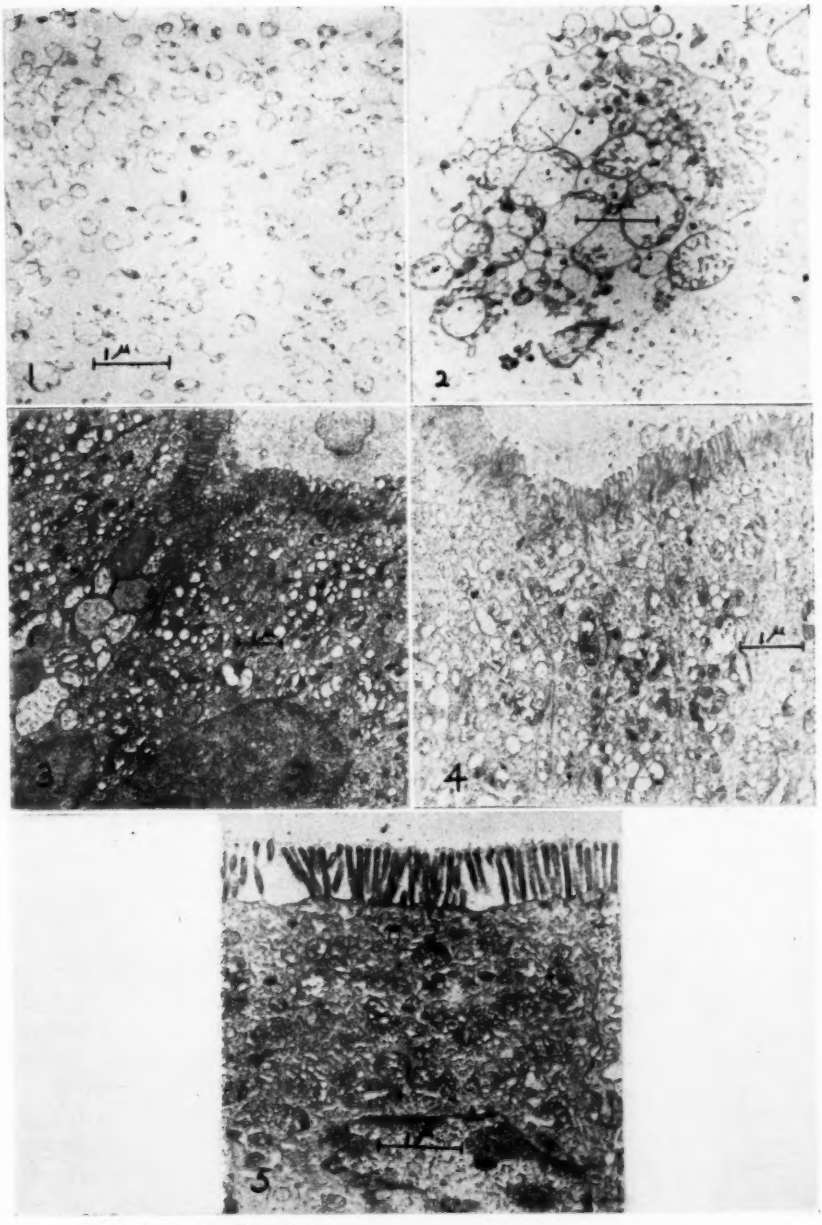

FIG. 1. Enterolipomicrons.
FIG. 2. Portion of disintegrating cell found in enterolipomicron fraction.
FIG. 3. Resting jejunal mucosa, minimal stimulation.
FIG. 5. Jejunal mucosa after repeated and prolonged stimulation.

Figs. 3 and 4 with Fig. 5. The mucosa of Fig. 5 is from a dog whose intestinal loop had been stimulated daily for a week by feeding followed by ½-hour distention of the loop. It will be noted that but few of the enterolipomicron-like particles are present and that the mitochondria appear to be numerous. In the specimens shown in Figs. 3 and 4, the loops had not been touched for a week and the specimens were taken with a minimum of manipulation. However, the dogs were accustomed or conditioned to us and the mere removal of the animal from the cage to the laboratory table undoubtedly resulted in a reflex stimulation. It will be noted that in these specimens there are many enterolipomicron-like particles in the cytoplasm and that some of them are seen to be passing out of the cell to the lumen. Relatively few mitochondria are seen. A logical interpretation of these findings is that the particles are accumulated in the cell during rest and are mobilized, in the manner of secretory granules, into the lumen during stimulation.

Debris produced during stimulation of the loop was also examined. As would be expected, many enterolipomicron-like particles are seen in the debris as well as unidentifiable material and a number of cells. Most of the cells were in a state of partial disintegration but some were practically intact. These cells reveal small bodies in the cytoplasm similar to enterolipomicrons as well as the mitochondria and other particles regularly seen in the cells.

Discussion

It seems clear that the enterolipomicrons are not chylomicrons derived from blood. From their composition, their enzyme activity, and their electron microscope appearance they would appear to be cytoplasmic granules. They are found in large numbers in the duodenal or jejunal lumen only during periods of digestive activity, and may, therefore, be considered as a secretory product. They are not obtained from ileum (two animals).

At the same time that these particles appear in the lumen a mass of cellular material also appears, but the amount of cellular debris does not always parallel the yield of enterolipomicrons. Furthermore, if the debris is homogenized and recentrifuged, the enteromicron-like particles remaining in the supernatant differ somewhat from those obtained normally by stimulating the intestinal loop. They are more irregular in size and shape, they have a somewhat lower lipid content, their alkaline phosphatase activity is lower, and their catalase activity is higher.

The varying proportions of debris and enterolipomicron fractions in different experiments, and the differences in composition between enterolipomicrons and the particles obtained from deliberate homogenation of the debris can logically be explained in two ways. These findings could result from varying degrees of homogenation taking place in the collection and handling of the specimens, assuming that a mild degree of manipulation will liberate from the cell a different fraction than will more severe handling. No doubt this explanation is true in part at least. On the other hand the findings can also be explained by

assuming that the enterolipomicrons are formed within the mucosal cell and discharged during physiological activity into the intestinal lumen. The enterolipomicron fraction as normally obtained would then be a mixture of these specific particles and of particles derived from disintegrating desquamated mucosal cells.

During the period of intestinal stimulation the cytoplasmic particles disappear from the mucosal cells and in some preparations can be seen to be leaving the cell. After prolonged stimulation very few of the particles are to be found within any of the cells. After minimal stimulation some cells contain large numbers of these microsomal bodies whereas other cells contain none. Could it be that the cells discharge their granules in an 'all-or-none' fashion?

This is pure speculation, of course, but the appearance of these preparations does suggest that some, at least, of the particles may leave the cell while it is still lining the mucosal surface.

Conclusions

The enterolipomicrons as ordinarily obtained are cytoplasmic particles derived from mucosal cells. They show enzymatic activity and are probably an important component of intestinal juice. Some, perhaps most, are liberated with the microsomal fraction of disintegrating desquamated cells, but some are extruded from the mucosal cells during digestive activity and may represent a true merocrine secretion.

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THE EFFECT OF CORTICOTROPIN ON THE FORMATION OF CORTICOSTEROIDS IN VITRO[1]

R. F. M. BAKKER AND D. DE WIED

Abstract

The effect of endogenous corticotropin on the formation of corticoids in vitro was evaluated under different experimental conditions.

Corticotropin, added in vitro to the adrenals of rats exposed to a strange environment, only moderately increased corticoid production, whereas the same amounts of corticotropin markedly stimulated the steroid formation of adrenals of "non-stressed" and hypophysectomized rats. Thus, the presence of endogenous corticotropin apparently interfered with the activity of added hormone.

The adrenal response to corticotropin after preincubation was more pronounced than that of non-preincubated glands. It appeared that the preincubation medium contained corticotropin-inactivating material which could be destroyed by heating. This suggests that the preincubation medium contains an enzyme that inactivates corticotropin. Presumably, this enzyme leaks into the medium from the cut surface of the adrenal gland.

Corticotropin stimulates the production of corticoids in vitro. The sensitivity of adrenal tissue to corticotropin is greatly increased by preincubation for 1 hour without added corticotropin (1). No explanation of the effect of preincubation has been provided as yet, although it was suggested by Saffran and Bayliss (2), that the tissue needs some time to recover from any previous stimulation by endogenous corticotropin. Earlier work has shown that the mere handling and transfer of rats from one room to the other results in a considerable increase of plasma corticoids (3) and of corticoid formation in vitro (4). Because the in vitro bioassay of corticotropin (stimulation of steroid output) is performed on adrenals from intact rats, it has been considered possible that the relative unresponsiveness of the adrenal gland in vitro during preincubation is due, at least partly, to the presence of endogenous corticotropin in the adrenals.

Accordingly, the effect of corticotropin has been studied on corticoid production in vitro of adrenals from rats placed in a new environment and that of adrenals of non-stressed and hypophysectomized animals.

This report suggests that the decreased sensitivity of adrenal glands in vitro is due partly to the continuing presence of endogenous corticotropin and partly to the activity of hormone-inactivating material, possessing enzymatic activity, released by the quartered adrenal tissue into the preincubation medium.

Materials and Methods

In all experiments, male rats of a Wistar strain weighing from 110 to 160 grams were used. Non-stressed rats were obtained by taking only two rats from a cage containing approximately 20 animals. These two rats were killed

[1]Manuscript received April 20, 1960.
Contribution from the Department of Pharmacology of the University of Groningen, the Netherlands.

by decapitation within 30 seconds after removal. It was assumed that this rapid procedure reduced the pituitary response to handling to a minimum. Animals subjected to handling and environmental change were used as "stressed" rats. After they had been placed into a cage, they were brought from the rat room to the operating room and thus subjected for at least 15 minutes to the strange environment (smell of blood and anesthetics, noise of animals and instruments, etc.). They were sacrificed by decapitation. Hypophysectomy was performed by the parapharyngeal approach under ether anesthesia. The "operated" animals were used 24 hours later. After sacrifice adrenal glands were removed quickly, dissected clean, quartered, and subsequently placed on a moist filter paper which covered an inverted Petri dish. Four adrenals of two animals were placed on the dish and four quarters, one from every adrenal, were weighed together and placed in a Warburg flask. This contained 2 ml of Krebs–Ringer–bicarbonate–glucose (200 mg%) solution.

Thus prepared adrenals were shaken for 1 hour at 37° C under oxygen containing 5% carbon dioxide ("preincubation"). In some experiments the flasks were recharged with fresh medium and the adrenals were incubated for another 1½ hours under the same conditions ("incubation").

After aspiration of the medium, the corticoids were extracted with 2 ml of methylene chloride. The absorption of ultraviolet light of the extract was determined spectrophotometrically. The difference in optical density between 240 and 260 mμ served as an index of the corticosteroid content of the methylene chloride extract.

The steroid content was calculated in μg per 10 mg adrenal tissue from a corticosterone calibration curve.

U.S.P. Standard Corticotropin* dissolved in Krebs–Ringer–bicarbonate–glucose medium was used throughout the experiment.

Results

In the first experiment, the effects of placing six rats in the operating room and of hypophysectomy (six rats operated on 24 hours previously) on adrenocortical activity were investigated. The respective outputs of corticoids were 3.37 ± 0.32 and 1.79 ± 0.18 μg/10 mg tissue/90 minutes respectively while that of six non-stressed rats was 1.53 ± 0.12. The strange environment considerably increased the subsequent steroid formation rate in vitro, whereas the rate of production by adrenals of non-stressed rats equalled that of rats hypophysectomized 24 hours previously.

In the second experiment the effect of preincubation on adrenals of six stressed and six non-stressed rats was studied. No statistically significant difference was found between the steroid formation of the two groups of adrenals if preincubation preceded incubation; the values were 1.75 ± 0.12 and 1.57 ± 0.19 respectively.

In the third experiment the effect of different doses of corticotropin on the

*Generously supplied by N. V. Organon, Oss, Holland.

production of corticoids in vitro of stressed, non-stressed, and hypophysectomized rats was determined. The incubation time was 1½ hours. Due to limited facilities for incubation, only three values for each dose of ACTH were obtained at the same time. The mean values are pooled results of two replicate experiments for each group of adrenals. Table I summarizes the results obtained when different doses of corticotropin were added to the medium of non-preincubated adrenals of stressed, non-stressed, and hypophysectomized rats, and to the medium of preincubated adrenals of stressed rats.

As could be expected, corticoid formation of adrenal tissue (not treated with ACTH) of stressed rats was significantly higher than that of non-stressed and hypophysectomized rats. In addition, the stimulation of corticoid production by graded doses of corticotropin, added to the medium, was least with adrenal glands from stressed rats. The major difference among the three groups was in the sensitivity to corticotropin; the lowest dose of ACTH that produced a significant increase in corticoid secretion was 45 mU. for adrenals from stressed rats, 5 mU. for non-stressed, and 15 mU. for hypophysectomized rats. However, in the higher dose range (45–180 mU.), the responsiveness of the three groups was similar. Thus, although in the higher dose range, corticoid production of these three groups increased in the same order, the major difference among them was the sensitivity to corticotropin. The lowest dose that produced a significant increase over corticoid secretion of adrenal tissue not exposed to ACTH by adrenals of stressed, non-stressed, and hypophysectomized rats was 45, 5, and 15 milliunits (mU.) respectively.

However, if adrenals of stressed rats were preincubated for 1 hour, the lower doses of corticotropin stimulated corticoid formation to a degree similar to that found in the foregoing groups after addition of the three highest doses of the hormone.

To investigate whether the difference in sensitivity between preincubated and non-preincubated adrenals could be due to the presence of corticotropin-inactivating material, 15 mU. U.S.P. ACTH was added to the medium of adrenals after preincubation for 1 hour, and these were then incubated for another 1½ hours. The same amount of hormone was added to another group of quartered adrenals and the medium was replaced at the same time. As is summarized in Table II the effect of corticotropin added to the preincubation fluid was significantly less than that of the same amount added to the fresh medium ($P<0.01$).

In the fifth experiment 30 mU. U.S.P. corticotropin was incubated for 1 hour at 37° C in 2 ml fresh medium or in 2 ml preincubation medium obtained from the adrenals of hypophysectomized rats. From these solutions, 1 ml was added to preincubated adrenal tissue together with 1 ml Krebs–Ringer–bicarbonate–glucose and these subsequently incubated for a further 1½ hours.

From the data in Table III it is clear that corticotropin exhibited much less activity when exposed to preincubation medium than the same amount of hormone added to fresh medium ($P<0.01$).

TABLE I

The effect of different doses of corticotropin on steroid formation by rat adrenals

Treatment	U.S.P. ACTH, milliunits added to medium					
	0	5	15	45	90	180
Non-preincubated adrenals of:						
stressed rats	3.39±0.27*	3.85±0.32	4.33±0.33	4.52±0.45	5.78±0.60	6.55±0.61
non-stressed rats	1.33±0.08	1.61±0.08	1.95±0.11	3.15±0.28	4.47±0.53	5.60±0.45
hypophysectomized rats	1.55±0.11	2.02±0.23	3.05±0.29	4.95±0.45	6.05±0.70	6.82±0.73
Preincubated adrenals of:						
stressed rats	1.95±0.09	3.45±0.13	4.95±0.55	6.90±0.17	—	—

*μg/10 mg tissue/90 minutes; mean of six values ± standard error (S.E.).

TABLE II

The effect of ACTH incubated in fresh or preincubation medium on steroid formation by adrenals from "non-stressed" rats

	Adrenal tissue, incubated in:	
	Fresh medium	Preincubation medium
No ACTH added	2.48±0.29*	2.63±0.24
15 mU. ACTH added	7.06±0.62	4.71±0.31
Net effect	4.71±0.50	2.08±0.22

*μg/10 mg tissue/90 minutes; mean of six values ± standard error (S.E.).

TABLE III

The effect of preincubating ACTH on the formation of steroids by adrenals from stressed rats

	15 mU. ACTH, incubated for 60 minutes in:	
	Fresh medium	Preincubation medium of adrenals of hypophysectomized rats
No ACTH added	1.45±0.07*	1.70±0.11
Incubated ACTH added	4.81±0.58	2.58±0.19
Net effect	3.36±0.82	0.88±0.18

*μg/10 mg tissue/90 minutes; mean of six values ± standard error (S.E.).

Assuming that the preincubation medium may contain corticotropin-inactivating enzyme(s), the foregoing experiment was repeated in such a way that the fresh medium and the preincubation medium were heated to 60° C for 30 minutes prior to addition of corticotropin.

Table IV summarizes the results. As can be seen, the effect of corticotropin exposed for 1 hour at 37° C to heated preincubation medium of adrenals of hypophysectomized rats was similar to the effect of the hormone incubated in heated Krebs–Ringer–bicarbonate–glucose for the same time.

TABLE IV

The effect of preincubating ACTH in heated media on the formation of steroids by adrenals from stressed rats

	15 mU. ACTH, incubated for 60 minutes in:	
	Fresh medium heated to 60° C for 30 minutes	Preincubation medium of adrenals of hypophysectomized rats heated to 60° C for 30 minutes
No ACTH added	1.96±0.18*	2.13±0.17
Incubated ACTH added	4.50±0.20	5.20±0.51
Net effect	2.54±0.17	3.07±0.59

*μg/10 mg tissue/90 minutes; mean of six values ± standard error (S.E.).

In order to study the effect of ascorbic acid released into the medium during preincubation (8) on corticotropin activity, 15 mU. of corticotropin was incu-

bated for 1 hour at 37° C in 1 ml of Krebs–Ringer–bicarbonate–glucose and in same medium containing 20 μg of ascorbic acid per ml. This approximates the amount of vitamin C present in the preincubate. Both solutions were added to preincubated adrenals from intact stressed rats and the incubation was continued for another $1\frac{1}{2}$ hours. The steroid production by corticotropin incubated for 1 hour with Krebs–Ringer–bicarbonate–glucose amounted to 4.55 ± 0.2 whereas the production by the same amount of hormone exposed to the vitamin amounted to 4.35 ± 0.5 μg per 10 mg adrenal.

Discussion

The above results reveal that the rate of steroid formation of adrenals in vitro increased considerably after rats were exposed to a strange environment. This is in accord with the findings of Fortier (3) and Van der Vies *et al.* (4), concerning the increase in plasma levels of free corticosterone and the production of corticosteroids in vitro by adrenals of rats subjected to similar stimuli. Since environmental change induced an increase in the in vitro activity of adrenals from intact rats, which does not occur in hypophysectomized rats, this in vitro production of steroids must be due to the continuing presence of endogenous corticotropin. Thus, the lessened adrenal sensitivity of stressed rats to exogenous corticotropin may be caused at least partly by competition with endogenous corticotropin.

The effect of endogenous corticotropin apparently disappeared after preincubation as preincubated adrenal tissue of stressed and non-stressed rats produced equivalent amounts of steroids during subsequent incubation.

However, the effect of corticotropin on steroid formation by adrenals of non-stressed or hypophysectomized rats was still considerably less than the effect observed using preincubated adrenal glands. The data of Table II suggests that a substance that inhibits or inactivates corticotropin accumulates in the preincubation fluid. The effect of corticotropin was markedly inhibited if the preincubation fluid was not replaced by fresh Krebs–Ringer–bicarbonate–glucose medium. Also the activity of corticotropin was greatly diminished after incubation in preincubation medium only.

The corticotropin-inactivating effect of preincubation medium could be destroyed after heating to 60° C for 30 minutes. This suggests that the preincubate contains enzyme(s) with corticotropin-inactivating properties.

Another explanation for the antagonistic influence of preincubation media on corticotropin could be that the corticoids present in the preincubate exhibit a direct inhibitory effect on adrenal activity. Birmingham and Kurlents (5) demonstrated that ACTH-free incubation medium had an inhibitory effect on corticoid production when added to fresh adrenals. These authors provided evidence that corticoids present in the preincubation medium, when exposed to fresh adrenals at a concentration of 4–6 μg per flask, were capable of suppressing the steroid formation rate in vitro. In the present experiments corticoid concentration of the preincubation medium obtained from adrenal tissue of

hypophysectomized rats amounted only to 0.94 ± 0.04 μg per 2 ml. From this, 1 ml containing 15 mU. of corticotropin was added to preincubated adrenal tissue. Accordingly, it is difficult to explain the present results on the basis of a direct inhibitory effect of corticosteroids on adrenal activity. However, it is possible that the adrenocortical tissue of the strain of rats used in our experiments is more sensitive to the inhibitory effect of corticoids present in the medium than that used by the aforementioned authors (5).

Ascorbic acid is a substance that leaks into the medium (8). Since it has been shown that this heat-labile vitamin is capable of inactivating corticotropin (9) the presence of vitamin C in the medium could have been responsible for the observed loss of activity of the hormone. However, incubation of corticotropin together with ascorbic acid for 1 hour did not significantly affect the hormonal activity on the rat adrenal gland.

The experiments do not reveal which enzyme(s) are involved in the inactivation of corticotropin (5). Todd and Trikojus (6) failed to observe any inactivation of corticotropin by adrenal proteinase. Apparently enzyme(s), so far unidentified, released into the incubation medium, probably as a result of the destruction of tissue cells after quartering of the adrenal glands, are responsible for the corticotropin-inactivating property of the medium. For example, Schönbaum *et al.* (7) demonstrated the leakage of TPN-linked dehydrogenases from cut adrenal tissue.

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THE REACTION OF SULPHURIC ACID WITH LYSOZYME AND HORSE GLOBIN[1]

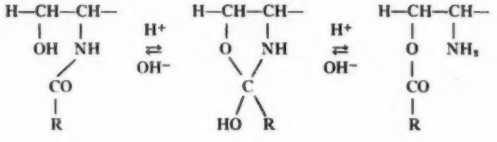
A. F. S. A. HABEEB[2]

Abstract

Lysozyme and horse globin have been treated with concentrated sulphuric acid to produce the N→O acyl shift. Results showed the reaction to be incomplete. Extensive chemical modification of the tyrosine, phenylalanine, tryptophan, and cystine residues occurs. Peptides obtained by degrading the modified protein with hydroxylamine could not be resolved either by paper chromatography or paper electrophoresis.

Introduction

Selective degradation of proteins utilizing concentrated sulphuric acid to produce an N→O acyl shift at threonine and serine as in the following equation has been carried out on silk fibroin (1), lysozyme (1, 2, 3), gluten (4), and gliadin (5). Peptides have been obtained from O-acyl protein by first blocking



the freed amino groups of β-hydroxy amino acids by deamination (5) or acetylation (1, 4) and then hydrolyzing with alkali to break the ester linkage. Recently Bock and Thakur (2, 3) used hydroxylamine to break the ester linkages in the O-acyl protein to liberate peptides from lysozyme, bovine serum albumin, and insulin. With the exception of Bock and Thakur (2, 3), who reported separation of the peptides by paper chromatography, others (e.g. Elliott (1)) found that the peptides obtained from lysozyme were completely nondiffusible. Peptides obtained from gluten (4) and gliadin (5) displayed a striking electrophoretic homogeneity which was attributed to strong association or chemical cross linking between peptide chains of the degraded protein.

Owing to the necessity of producing specific cleavage of protein molecules in sequence studies, a study of the reaction and effects of concentrated sulphuric acid on lysozyme and horse globin was carried out.

[1]Manuscript received July 29, 1960.
Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada.
Issued as N.R.C. No. 6080.
[2]N.R.C. Postdoctorate Fellow, 1959–1960. Present address: Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario, Canada.

Materials

L-Glutamine, DL-asparagine, and DL-tryptophan were obtained from Nutritional Biochemicals Corporation, tyrosine from Matheson Co., and lysozyme from Armour Research Division. Horse globin was prepared from fresh citrated blood (6). Salt-free hydroxylamine, 1 *M* (6 *N*), was prepared by adding 1 *M* barium hydroxide solution gradually with magnetic stirring to 1 *M* hydroxylamine sulphate solution to pH 7.2–7.3. The solution was filtered and normality adjusted by titration with potassium bromate. The method given by Thakur (3) was found to be inconsistent with the solubility properties of hydroxylamine hydrochloride and could not be duplicated. Sulphuric acid 98% and 100% were prepared by adding 5.9 and 11.8 g of fuming sulphuric acid, 15% SO_3 to 10 g of concentrated sulphuric acid respectively.

Methods and Results

Reaction of Sulphuric Acid with Asparagine and Glutamine

The increase in free amino groups due to N→O acyl shift could be conveni-

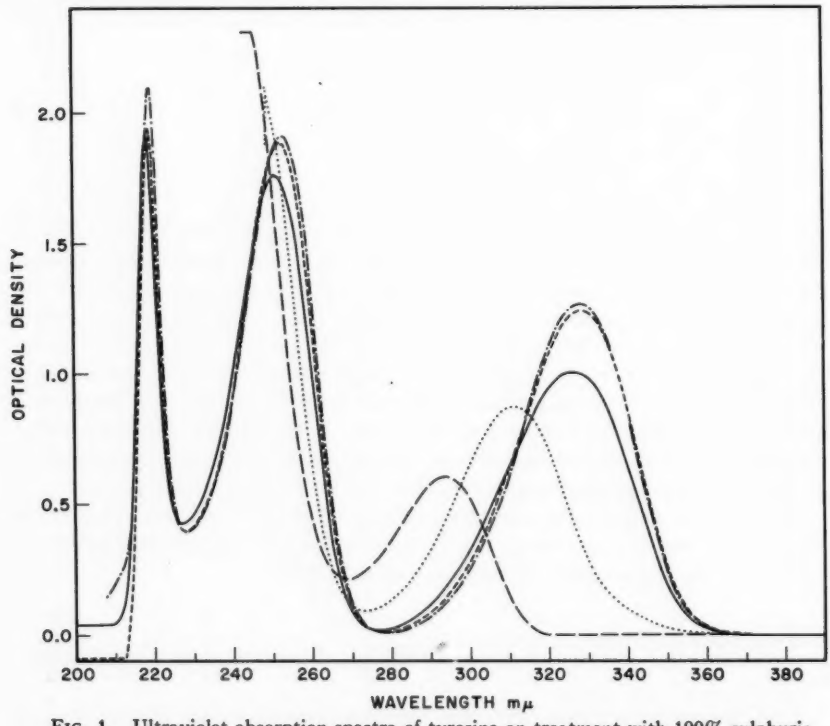


FIG. 1. Ultraviolet absorption spectra of tyrosine on treatment with 100% sulphuric acid; — — tyrosine, . . . ½-hour reaction, — 3-hour reaction, – – – 6-hour reaction, —·— 24-hour reaction.

ently followed by the ninhydrin color reaction, provided that no ammonia was liberated by the action of sulphuric acid on asparagine and glutamine.

To test for possible liberation, asparagine or glutamine was reacted in sulphuric acid 98% and 100% at room temperature. Aliquots were withdrawn at intervals and tested with ninhydrin reagent (7). No increase in the ninhydrin coloration was found up to 48 hours showing that the amide groups were stable towards sulphuric acid under these conditions.

Reaction of Sulphuric Acid with Tyrosine and Tryptophan

Tyrosine and tryptophan and also a mixture of both were treated with 98% and 100% sulphuric acid. At intervals aliquots were withdrawn. Ultraviolet absorption spectra of the solutions in sodium hydroxide were obtained in a Cary recording spectrophotometer. Figures 1, 2, and 3 show the change in ultraviolet absorption spectra of tyrosine and tryptophan and a mixture of both on treatment with 100% sulphuric acid. Tyrosine and tryptophan showed shifts in their maxima which were complete after 3 hours towards longer wave

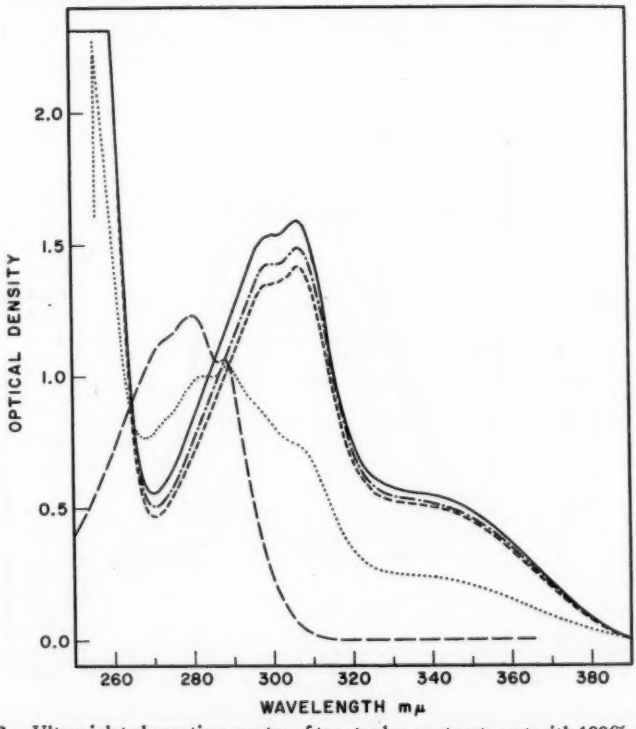


FIG. 2. Ultraviolet absorption spectra of tryptophan on treatment with 100% sulphuric acid; —— tryptophan, . . . ½-hour reaction, — 3-hour reaction, —·— 6-hour reaction, – – – 24-hour reaction.

lengths with the appearance of several U.V. absorption bands. An equimolar mixture of tyrosine and tryptophan treated with 100% sulphuric acid showed spectral shifts which, essentially, reached completion after about 6 hours reaction (Fig. 3), while that treated with 98% sulphuric acid showed shifts after 24 and 48 hours that were similar but not identical, showing that the chemical changes that produced these shifts were not complete.

The mixture of tyrosine and tryptophan after reaction with 98% sulphuric acid was precipitated by ether. An aliquot, subjected to paper chromatography on Whatman No. 1 paper using butanol – acetic acid – water system 3:1:1 by volume, showed five to six ninhydrin positive spots while the product of sulphuric acid treated tyrosine gave only one slightly elongated spot at point of application.

Reaction of Lysozyme and Horse Globin with Sulphuric Acid

To effect the N→O acyl shift other workers have used 97.5% (1) and anhydrous sulphuric acid (4, 5) and reaction times from 3–5 days (1–5) at

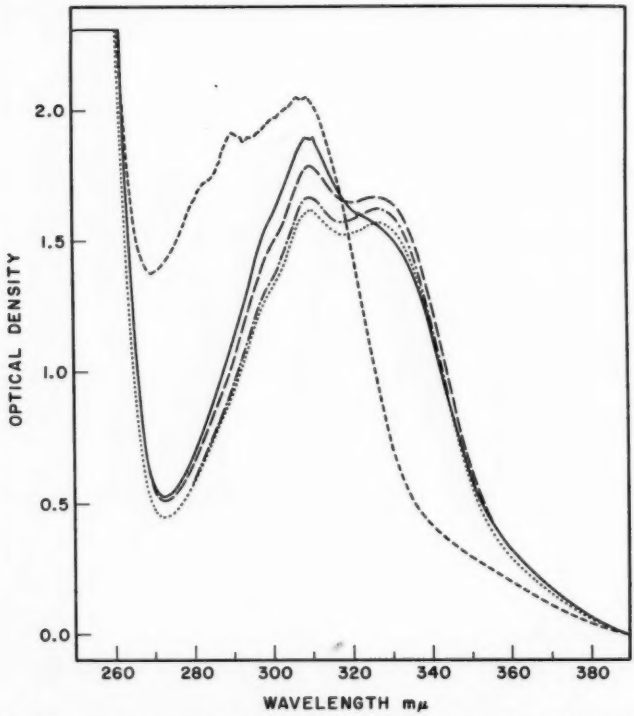


FIG. 3. Ultraviolet absorption spectra of a mixture of tyrosine and tryptophan on treatment with 100% sulphuric acid; – – – ½-hour reaction, — 3-hour reaction, — — 6-hour reaction, —·— 24-hour reaction, . . . 48-hour reaction.

room temperature. The course of the reaction was therefore investigated and the N→O acyl shift was followed by the appearance of free amino groups as tested by ninhydrin (7). The reaction was carried out by dissolving the protein at 2% concentration using 96%, 98%, and 100% sulphuric acid in the cold. The solution was brought to room temperature and stirred for 72 hours. At intervals, aliquots were withdrawn and tested with ninhydrin. Figures 4 and 5 show optical density of ninhydrin color due to free amino groups plotted vs. time for lysozyme and horse globin respectively. The increase after 24 hours is very little and slow.

To test whether degradation had taken place aliquots were withdrawn at 12-, 24-, 48-, and 72-hour intervals from the reaction mixture, and the protein was precipitated and washed with ether. It was taken up in water and an aliquot was subjected to paper electrophoresis on Whatman 3 MM paper using 1.2 *N* acetic acid at 450 v for 3–4 hours. The modified protein that had been treated with 96% and 98% sulphuric acid gave a trail that moved towards the cathode while that treated with 100% acid showed a compact spot that moved slightly towards the cathode with a trail above and below it. These results may point to heterogeneity of the modified protein but give no conclusive evidence for degradation.

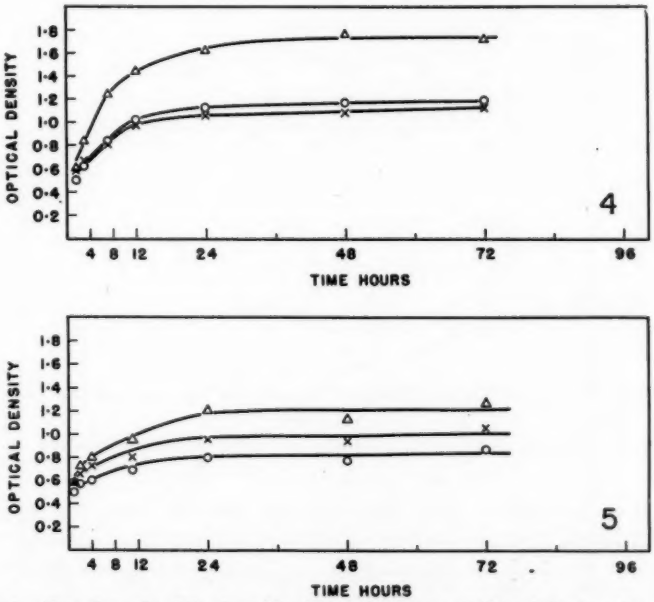


FIG. 4. Reaction of sulphuric acid with lysozyme; ○ 96% sulphuric acid, × 98% sulphuric acid, △ 100% sulphuric acid.

FIG. 5. Reaction of sulphuric acid with horse globin; ○ 96% sulphuric acid, × 98% sulphuric acid, △ 100% sulphuric acid.

O-Acyl proteins that were studied further were, unless otherwise mentioned, obtained by reacting the protein with 98% or 100% sulphuric acid for 24 hours at room temperature, after which the solution was cooled on solid carbon dioxide, and the protein was precipitated and washed with anhydrous ether until sulphate free. The protein was then lyophilized. The products obtained by reacting lysozyme and horse globin with 98% acid were not completely water-soluble but those obtained with 100% sulphuric acid dissolved.

Ultraviolet Spectra

O-Acyl lysozyme and O-acyl horse globin were dissolved in 0.2 *N* sodium hydroxide and the solution examined in the Cary recording spectrophotometer. Results are shown in Figs. 6 and 7 respectively and are characterized by significant shifts of their maxima and greater absorption at longer wave lengths.

Infrared Spectra

Samples of lysozyme, horse globin, and the corresponding O-acyl proteins were prepared in nujol mull and examined in the Perkin–Elmer 21 infrared recording spectrometer. Figure 8 shows the infrared spectra for lysozyme and

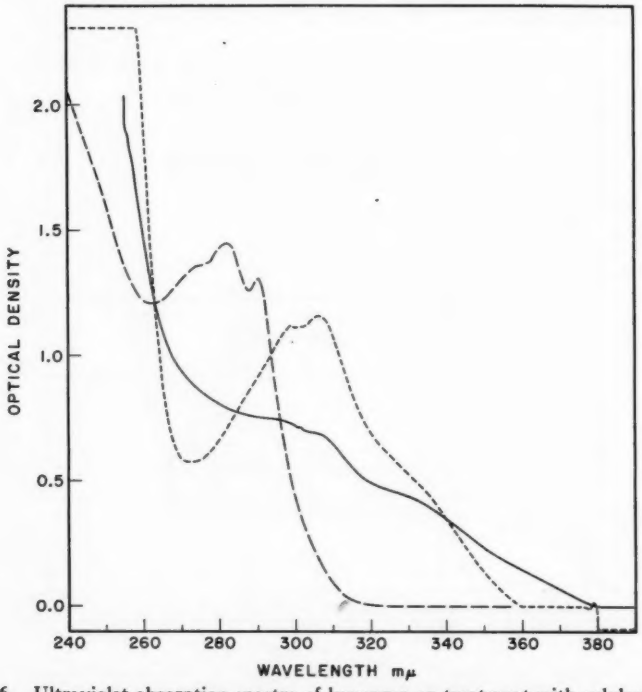


FIG. 6. Ultraviolet absorption spectra of lysozyme on treatment with sulphuric acid; —— native lysozyme, - - - treated with 98% sulphuric acid, — treated with 100% sulphuric acid.

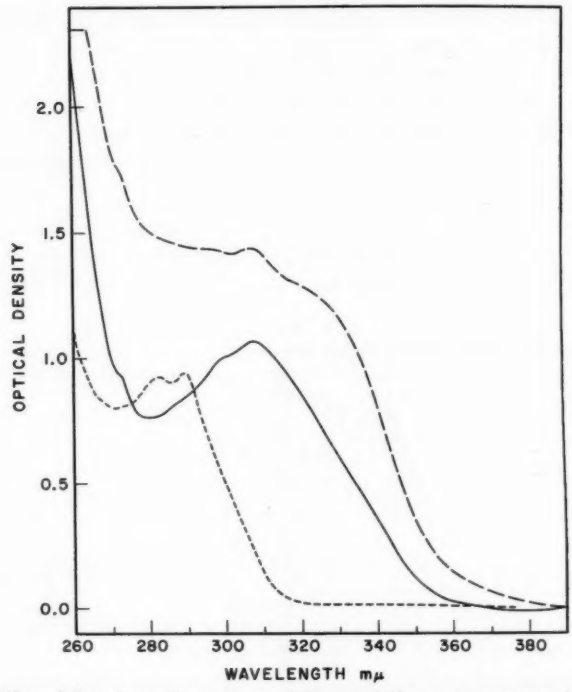


FIG. 7. Ultraviolet absorption spectra of horse globin on treatment with sulphuric acid; - - - native horse globin, — 98% sulphuric acid, — — 100% sulphuric acid.

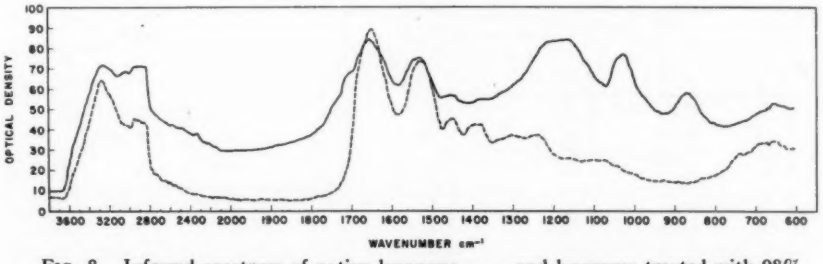


FIG. 8. Infrared spectrum of native lysozyme - - - and lysozyme treated with 98% sulphuric acid ——.

O-acyl lysozyme prepared with 98% sulphuric acid. There were no significant differences in the spectra of the modified proteins prepared with 98% or 100% sulphuric acid for 1 or 3 days.

Reaction of O-Acyl Proteins with Fluorodinitrobenzene (FDNB)

Lysozyme, O-acyl lysozyme, horse globin, and O-acyl horse globin were reacted with FDNB. The dinitrophenyl (DNP) protein was hydrolyzed with

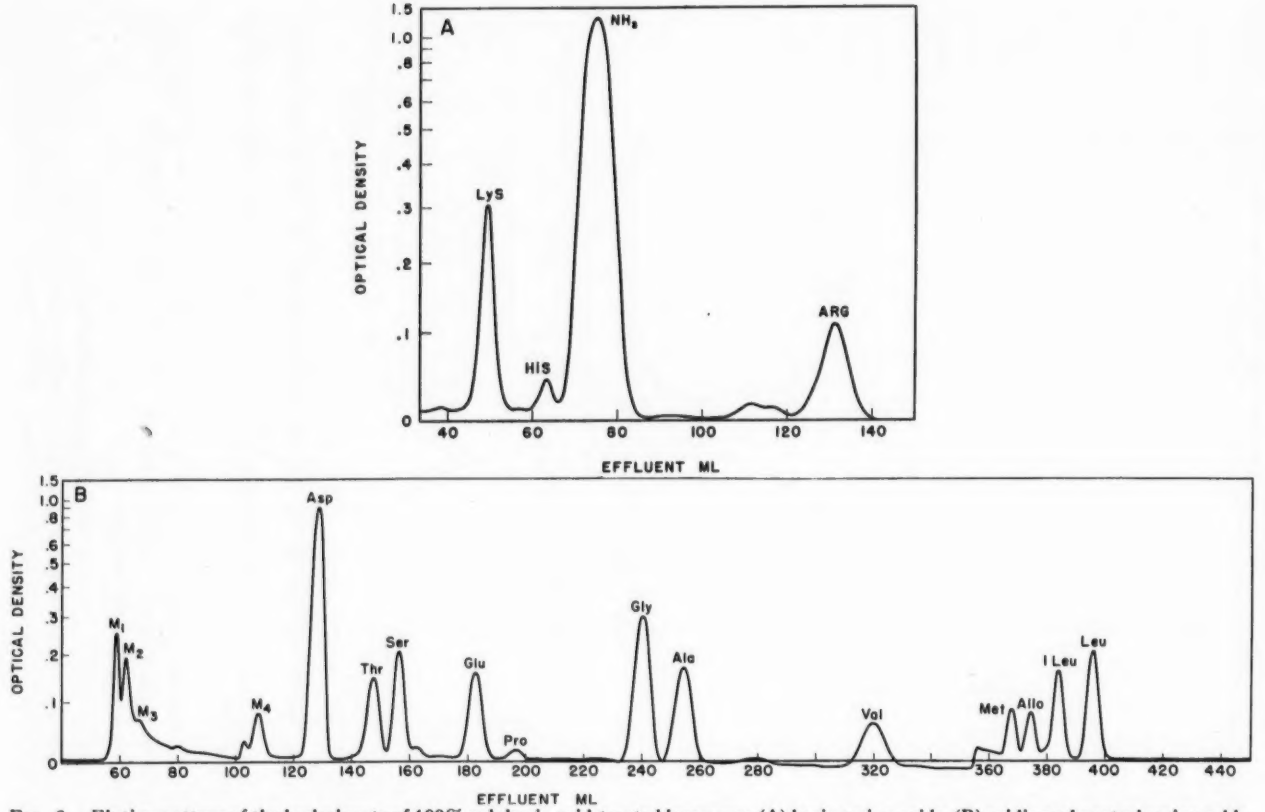


FIG. 9. Elution pattern of the hydrolyzate of 100% sulphuric acid treated lysozyme; (A) basic amino acids, (B) acidic and neutral amino acids.

6 *N* hydrochloric acid for 16 hours. The liberated DNP-amino acids were extracted with ether and separated by paper chromatography using the ethyl benzene system (8). The excised spots were extracted with 1% sodium bicarbonate solution, and optical density readings at 360 mμ were converted to molar ratios. Aspartic acid was separated from glutamic acid and identified by the tert-amyl alcohol – phthalate buffer system (9). Results are given in Table I.

Amino Acid Analysis of Proteins

Native and modified proteins were hydrolyzed in sealed evacuated tubes with 6 *N* hydrochloric acid at 105° C. Analyses using the Beckman Spinco

TABLE I

N-Terminal amino acids in native and sulphuric-acid-treated proteins*

Protein	Mole amino acid/mole protein				
	N-terminal	Serine	Threonine	Aspartic	Others (traces)
Horse globin	Val 2.9	—	?	—	gly
Horse globin (98% H_2SO_4)	Val 1.6	1.2	0.42	0.23	ala and glu
Horse globin (100% H_2SO_4)	Val 1.5	0.46	0.40	0.26	ala and glu
Lysozyme	Lys 0.84	—	—	—	—
Lysozyme (98% H_2SO_4)	Lys 0.45	0.46	0.39	0.09	ala ?, glu
Lysozyme (100% H_2SO_4)	Lys 0.28	0.08	0.08	0.08	ala ?, glu

* Not corrected for moisture in protein nor for destruction during hydrolysis.
? Doubtful.

TABLE II

Amino acid content of native and sulphuric acid treated proteins

Amino acid	Lysozyme, mole amino acid/mole protein			Horse globin, mole amino acid/mole protein		
	Native	98% H_2SO_4	100% H_2SO_4	Native*	98% H_2SO_4	100% H_2SO_4
Lysine	6.4	6.0	4.8	43.5	40.0	38.4
Histidine	1.06	0.77	0.81	35.4	34.3	34
Arginine	10.9	9.70	4.3	13.8	13.0	11.1
Aspartic acid	20.0	20.0	20.0	56.5	54.0	54.9
Threonine	6.2	5.7	3.7	22.6	21.3	19.4
Serine	7.1	6.4	4.3	32.3	32.9	30.7
Glutamic acid	4.6	4.6	4.5	36.9	37.9	37.9
Proline	2.0	2.3	2.0	20.3	24.8	22.0
Glycine	11.7	11.4	9.9	51.7	47.8	46.2
Alanine	11.8	11.6	5.9	63.5	59.3	55.6
Valine	4.2	4.6	3.8	58.2	43.8	41.7
Methionine	2.2	1.7	1.6	4.5	1.2	3.6
Isoleucine	4.3	4.6	3.2	0	0	0
Leucine	7.3	7.1	4.6	84.3	76.4	74.6
Tyrosine	3.2	0	0	12.5	0	0
Phenylalanine	2.7	0	0	30.4	0	0
Alloisoleucine	0.4	0.4	1.4	0	0	0
Cystine/2	6.1	4.5	0	0	0	0
M_1	0	1.3	3.1	0	4.8	2.3
M_2	0	3.6	3.1	0	26.3	27.1
M_3	0	4.4	3.8	0	12.3	9.3

*Values from reference (11) for 24-hour hydrolysis.

FIG. 9. Elution pattern of the hydrolyzate of 100% sulphuric acid treated lysozyme; (A) basic amino acids, (B) acidic and neutral amino acids.

amino acid analyser (10) are given in Table II. Figure 9 shows the elution pattern of N→O acyl lysozyme prepared with 100% sulphuric acid. Peaks M_1, M_2, M_3 were common to all proteins treated with sulphuric acid.

Degradation of O-Acyl Proteins with Hydroxylamine

O-Acyl lysozyme (50 mg) was stirred with 5 ml 1 *M* (6 *N*) of hydroxylamine solution pH 6.8; aliquots were withdrawn at intervals and tested with ferric chloride solution. It was found that the hydroxamic color test reached a maximum in 24 hours. After reaction the solution was lyophilized.

Degradation of O-acyl horse globin was carried out by reaction for 2 hours at pH 7.2–7.3 at 37° C and then for a further hour at pH 8 (12), and then the solution was lyophilized.

The degraded protein was subjected to paper chromatography using butanol – acetic acid – water system 3:1:1 by volume. The chromatogram was treated by chlorine – starch – potassium iodide (13) as the color with ninhydrin was very weak. No peptides were resolved as only a spot at point of application with a trail above it showed. When the degraded protein was subjected to paper electrophoresis on Whatman 3 MM using 1.2 *N* acetic acid, it showed a streak from point of application extending towards both the cathode and the anode and resolution was poor.

Discussion

The ultraviolet spectral changes of tyrosine and tryptophan on treatment with sulphuric acid are evidence of chemical modification of these amino acids. Thus sulphonation of tyrosine and substitution and degradation of the indole nucleus in tryptophan is probable. Reitz *et al.* (14) showed that tyrosine forms the sulphonate with only traces of sulphate after 1-hour reaction with sulphuric acid. Paper chromatographic analyses of the reaction products gave one spot with tyrosine assumed to be tyrosine sulphonate and about five spots with tryptophan.

Figures 4 and 5 showed that N→O acyl shift as evidenced by increase in ninhydrin color reached a maximum after 24 hours at room temperature. This is a shorter reaction time than the 3–5 days recommended by other workers (1–5). Table III gives the increase in N-terminal groups as calculated from

TABLE III

% increase in N-terminal groups from ninhydrin on treating lysozyme and horse globin with sulphuric acid

Sulphuric acid	Lysozyme		Horse globin	
	Found %	Theoretical %	Found %	Theoretical %
96%	133	143*	67	96*
98%	112		70	
100%	226	243†	116	129†

*If all serine residues have undergone N→O acyl shift.
†If all serine and threonine residues have undergone N→O acyl shift.

ninhydrin color yield for lysozyme and horse globin. The results show that 96% and 98% sulphuric acid do not effect complete N→O acyl shift while with 100% acid it approaches the theoretical. Elliott (1) found that with lysozyme all the serine and one-third of the threonine participated in the reaction. Thakur (2) found the serine:threonine ratio to be 2.5:1.

Significant changes in the U.V. absorption spectra of O-acyl proteins is evidence of changes in their aromatic amino acids.

In the infrared spectra (Fig. 8), the main bands due to peptide linkages (15, 16) were common to all proteins (native and sulphuric acid treated). These included a band at 3270 cm^{-1}, 1650 cm^{-1}, and 1530 cm^{-1}. With lysozyme the band at 3450 cm^{-1} reported by Klotz *et al.* (17) was absent. With horse globin two weak bands were observed at 1230 cm^{-1} and 1170 cm^{-1}. A shoulder on the amide I band at about 1710 cm^{-1} may be assigned to the unionized carboxyl group, since Ehrlich and Sutherland (18) observed the appearance of a shoulder at 1710 cm^{-1} if films of bovine serum albumin and β-lactoglobulin were cast from hydrochloric acid solution. Since horse globin was prepared by precipitation from oxyhemoglobin in acetone – hydrochloric acid solution, this band is to be expected. The band at 1230 cm^{-1} may be assigned tentatively as a carboxylic band originating in a C—O stretching or OH deformation mode (18).

O-Acyl proteins showed, in addition to the bands due to the peptide links, the following: (*a*) A shoulder at 1710 cm^{-1} which may be assigned to unionized carboxyl groups, which would be expected since the proteins were precipitated with ether from sulphuric acid solution. (*b*) A shoulder that appears at 1740 cm^{-1} is assigned to C=O stretching vibration of ester. This is supported by the fact that saturated esters exhibited absorption (19) close to 1740 cm^{-1} and also by the finding that poly-DL-serine treated with sulphuric acid (20) showed the appearance of an ester band at 1740 cm^{-1}. The appearance of this shoulder at 1740 cm^{-1} is a qualitative proof of N→O acyl shift. (*c*) The appearance of two strong bands with maxima at 1200 cm^{-1} and 1030 cm^{-1} which are assigned to sulphonate. Bellamy (21) examined a number of sulphonic acids and salts and found consistently the 1200–1160 cm^{-1} and the 1060–1030 cm^{-1} bands.

The recovery of DNP-serine and -threonine from hydrolyzates of DNP-derivative of O-acyl proteins (Table I) is evidence that the N→O acyl shift occurs. It gives no quantitative measure of the extent of shift since the N→O rearrangement is reversible at alkaline pH. The presence of aspartic acid and a trace of alanine and glutamic acid may indicate non-specific fission. The decrease in the yield of the N-terminal amino acids in proteins treated with sulphuric acid may reflect increased destruction of DNP-amino acid in presence of tyrosine sulphonic acid or degradation products of tryptophan.

The amino acid analyses of lysozyme and horse globin treated with 98% sulphuric showed the disappearance of all the tyrosine and phenylalanine probably due to sulphonation of the benzene ring accompanied by the appearance of three acidic peaks, M_1, M_2, and M_3 (Fig. 9 and Table II). M_2 and M_3 could be assigned on quantitative reasons to phenylalanine sulphonic acid and

tyrosine sulphonic acid. M_1 and part of M_3 may be acidic degradation products of tryptophan. A band M_4 appeared in the hydrolyzate of 100% sulphuric acid treated lysozyme and was accompanied by the disappearance of cystine; this may be a thiosulphate resulting from the action of sulphuric acid on cystine. Cystine suffers some destruction but other amino acids were not greatly affected. Lysozyme treated with 100% sulphuric acid shows decreases in some of its amino acids, especially cystine, arginine, and alanine. The peak at the position of alloisoleucine may be assigned tentatively to this amino acid in spite of its size (Fig. 9). Increased racemization of isoleucine may occur either during hydrolysis of 100% sulphuric acid treated lysozyme or during reaction of the protein with sulphuric acid.

Horse globin treated with 100% sulphuric acid showed similar decreases in several of its amino acids but they were not as marked for arginine and alanine as with lysozyme. Methionine, arginine, tyrosine, tryptophan, cystine, and phenylalanine had been found to decrease in sulphuric acid treated gliadin (5).

In contrast to results of Bock and Thakur (2, 3) the peptides obtained from degraded protein could not be resolved by paper chromatography. Interference due to salt was suggested (12). Hydroxylamine of pH 7.2 will contain some hydroxylamine sulphate which will remain with the degraded protein after lyophilization. However, this residual salt did not interfere in the separation and resolution of added amino acids or peptides from a tryptic digestion of horse globin when chromatographed with the degraded protein. Results indicate that peptide hydroxamates have solution properties different from peptides. Thakur found that they would not move in paper electrophoresis. Peptides obtained from lysozyme (1), gluten (4), and gliadin were reported not to be resolved.

The results obtained in this work show that the incompleteness of the N→O acyl shift and the extensive chemical modification of several of the amino acids, with the destruction of some, makes sulphuric acid treatment of limited value as a reagent for selective fission of peptide links. For sequence study of proteins the integrity of amino acid residues is very important.

Acknowledgments

I wish to thank Dr. David B. Smith for his advice throughout the work and Mr. J. Labelle for performing the amino acid analyses.

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A STUDY OF THE PERMEABILITY BARRIER TO β-GALACTOSIDES IN ESCHERICHIA COLI B[1]

R. HOLMES,[2] ROSE SHEININ,[3] AND BRUCE F. CROCKER

Abstract

Investigation of the β-galactosidase activity of *Escherichia coli* B has confirmed the view that the access of β-galactosides to the intracellular site of enzyme activity is limited by a permeability barrier. Evidence presented indicates that passage of β-galactosides across this barrier is effected by the combined action of a process of physical diffusion and that of an enzyme-like transport system.

Introduction

The work of Deere, Dulaney, and Michelson (1) and that of Lederberg (2) demonstrated that the activity of the enzyme hydrolyzing β-galactosides, now widely known as β-galactosidase (3, 4), in *Escherichia coli* was much less in intact cells than that measured with cells which had been treated so as to disrupt cellular integrity. These observations have been repeatedly confirmed (5, 6, 7, 8, 9). Lederberg concluded that intact cells exhibited a lower activity because the enzyme was situated within a permeability barrier which limited the rate of entrance of substrate to the site of enzyme action. It would be expected that an understanding of the mechanism of permeation of this barrier would provide answers to particular problems associated with the activity of β-galactosidase and with the induction of its synthesis.

To gain such an understanding, studies were initiated in various laboratories. A major contribution was made by Cohen, Rickenberg, Buttin, and Monod (10, 11, 12). These workers demonstrated the existence in *E. coli* of a specific permeation system for β-galactosides, which they termed "galactoside permease". In a study of a cryptic mutant of *E. coli* ML 30, Herzenberg (13) reported that these organisms are devoid of the "permease" and that entry of substrate takes place by passive diffusion. His studies suggest that diffusion is operative in the wild type organism as well. The studies of Rotman (9, 14) have provided further evidence for a specific permeation mechanism for β-galactosides. Two independent permeation systems, one of which transports methyl-β-D-galactoside and the other methyl-β-D-thiogalactoside have been reported by Rotman (14). Investigations in this laboratory (15) provide evidence concerning the mechanism of transport of β-galactosides into *E. coli* B. These studies indicate that permeation of the cell is effected through the combined action of physical diffusion and of an enzyme-like transport system.

[1]Manuscript received June 27, 1960.
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Materials and Methods

Organism

Experiments were performed with *Escherichia coli* B. Organisms were grown in either the tryptose or S5 medium, described below, to mid-logarithmic phase and were then lyophilized to provide enough material for approximately 6 months (7).

Media

Tryptose medium contained tryptose (Difco, 2% w/v), NaCl (0.5% w/v), and glucose (0.1% w/v). S5 medium contained L-glutamic acid (British Drug Houses, 0.5% w/v); glycine (British Drug Houses, Analar, 0.2% w/v); glycerol (redistilled, 0.2% w/v); KH_2PO_4 (0.14% w/v); $MgSO_4 \cdot 7H_2O$ (0.002% w/v); $CaCl_2$ (0.001% w/v); and $FeSO_4 \cdot 7H_2O$ (0.05 mg% w/v). These media were adjusted to pH 7.4 with KOH.

Cultivation of Bacteria

Lyophilized bacteria were suspended in 30 ml tryptose medium (NaCl omitted) and rocked in L tubes for 24 hours at 25° C. A portion of the now exponentially growing culture was subcultured into the same medium and grown for 15 hours at 25° C.

Induction of β-Galactosidase Synthesis

Bacteria, grown as described above, were harvested by centrifugation, washed with and resuspended in potassium phosphate buffer (0.05 *M*, pH 7.0) to a dry weight of 0.76 mg per ml. The cell suspension was determined quantitatively from a standard curve relating dry weight of organism to the photometric density measured with an Evelyn colorimeter at 420 mμ. A 6-ml portion of the above suspension was mixed in an L-shaped tube with 10 ml of an induction mixture containing inducer, yeast extract (Difco, 1.3% w/v), and sodium succinate (1.8% w/v) in 0.05 *M* potassium phosphate buffer, pH 7.05, and rocked in a water bath at 37° C. To halt enzyme formation, a sufficient volume of a solution of chloromycetin (Parke, Davis and Co.) to produce a final concentration of 20 μg/ml was added. The cells were harvested and washed twice with and resuspended in sodium phosphate buffer (0.05 *M*, pH 7.05) containing 20 μg/ml of chloromycetin so that the cell concentration corresponded to 0.12 mg dry weight/ml. These cell suspensions were stored at 2° C pending measurement of enzyme activity.

Measurement of β-Galactosidase Activity

The β-galactosidase activity of either untreated (intact) or toluene-treated cell preparations (7) was measured with *o*-nitrophenyl-β-D-galactopyranoside as chromogenic substrate (2) at a concentration of 3.2×10^{-3} *M*, unless otherwise noted, and according to the procedure described by Koppel *et al.* (7). Chloromycetin (20 μg/ml) was present in the substrate solution. β-ONPG* was synthesized according to the procedure of Seidman and Link (16). The β-galactosidase activity of intact cells was always measured within 12 hours

*See footnote p. 47.

of the preparation of their suspensions (after approximately 16 hours at 2° C the cells began to lyse and release free β-galactosidase). The activity of such cell suspensions was constant with time for 2 to 5 minutes. A curve relating activity and time was plotted from measurements made at 30-second intervals for 5 minutes. The initial velocity of enzyme action was calculated from the linear portion of this curve. β-Galactosidase activities are expressed as μmoles *o*-nitrophenol released/minute/mg bacterial nitrogen as determined by a standard micro-Kjeldahl method. The ONP* released was determined from a standard curve relating concentration of ONP to the photometric density measured at 420 mμ with an Evelyn photoelectric colorimeter.

Results

To learn something of the mechanism(s) by which galactosides enter the *E. coli* B cell the properties of β-galactosidase, as exhibited by intact and by toluene-treated cell preparations, were examined.

Relation between β-Galactosidase Activity and the Concentration of Its Substrate

The results of a study of the relationship between the β-galactosidase activity of induced cells of *E. coli* B and the concentration of substrate are presented in Fig. 1.

The activity, as measured with toluene-treated cells, was found to follow

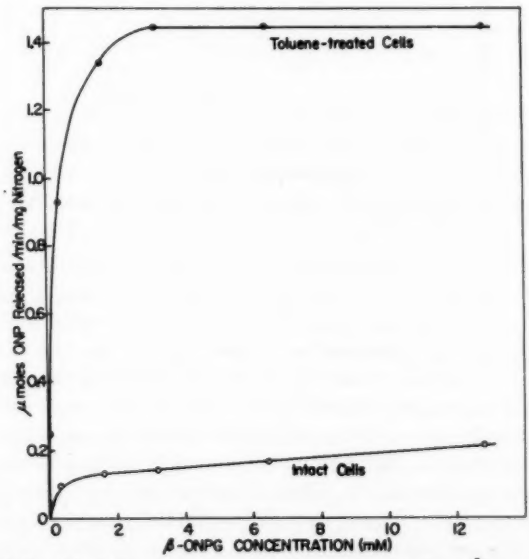


FIG. 1. Relationship between β-galactosidase activity of *E. coli* B and external substrate concentration.

*Abbreviations used: β-ONPG: *o*-nitrophenyl-β-D-galactoside; ONP: *o*-nitrophenol; β-TMG: methyl-β-D-thiogalactoside.

normal Michaelis–Menten kinetics (17), approaching a maximum at a substrate concentration of approximately 3.2×10^{-3} *M*. The curve for the enzyme activity of intact cells rose in a hyperbolic fashion up to a substrate concentration of approximately 1.6×10^{-3} *M*. Above this concentration, however, there was a linear relation between the observed activity and the concentration of β-ONPG. The shape of this latter curve suggested that two distinct processes were involved in the activity of intact *E. coli* B cells. This suggestion is seen more clearly when the data are replotted according to the method of Lineweaver and Burk (18) as shown in Fig. 2.

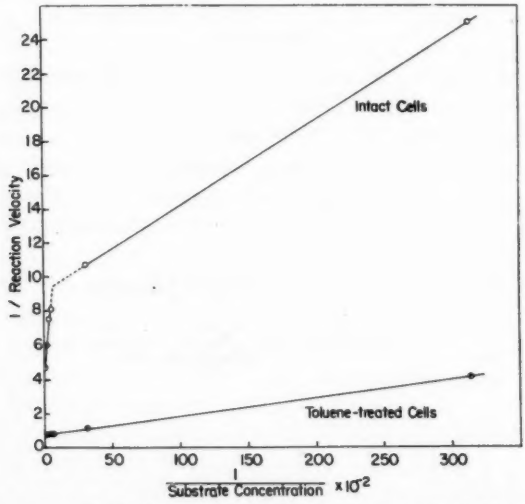


FIG. 2. Lineweaver–Burk plot of relationship between β-galactosidase activity of *E. coli* B and substrate concentration.

It is clear that, over the entire range of concentrations, the β-galactosidase activity of toluene-treated *E. coli* B is limited by a single reaction with a Michaelis constant (K_M) for β-ONPG of 1.7×10^{-4} *M*. This value is very nearly the same as that obtained by Lederberg (2) for the same enzyme in benzene-treated *E. coli* K12 and by Kuby and Lardy (19) for the partially purified enzyme, indicating that in measuring the activity of toluene-treated cells one is measuring the activity of β-galactosidase per se.

The discontinuous plot obtained with intact cells indicated that two distinct reactions limit the observed β-galactosidase activity in different ranges of substrate concentration. The hyperbolic portion of the curve relating activity and substrate concentration obtained with intact cells (Fig. 1) suggested that, in the range of substrate concentrations up to 1.6×10^{-3} *M*, the predominating reaction responsible for the observed activity might be enzymic in nature. In the range of concentrations above this level the linear relationship between

activity and substrate concentration indicated that, over this range, a process of physical diffusion predominated in determining the observed activity.

If diffusion is involved in the β-galactosidase activity exhibited by intact *E. coli* B cells, it would be so involved over the entire range of substrate concentrations employed. This component of the observed activity could be represented by a line (curve 2, Fig. 3) passing through the origin parallel to the

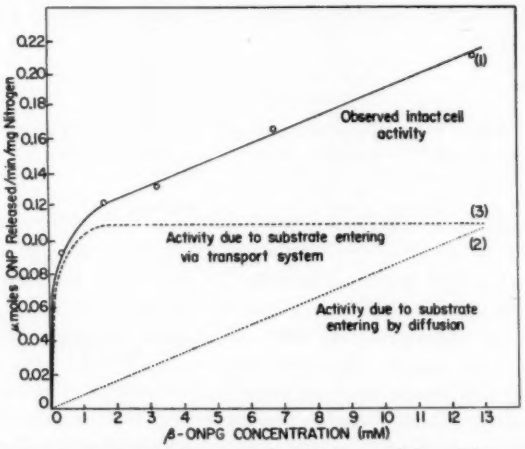


FIG. 3. The two components of the β-galactosidase activity of intact *E. coli* B preparations.

linear portion of the experimentally determined curve. Subtraction of activity due to this diffusion process from the observed activity would permit one to calculate the activity mediated by the postulated enzymic transport system (curve 3). The curve so obtained followed Michaelis–Menten kinetics and permitted calculation of an "apparent" K_M of 3.8×10^{-5} *M* for β-ONPG and an "apparent" saturation concentration of 1.6×10^{-3} *M* β-ONPG for this system.

Inhibition of β-Galactosidase Activity by Sugars

The effects of the presence of each of a number of sugars on the β-galactosidase activity of induced *E. coli* B are shown in Table I. The sugars were dissolved in the substrate solution. In all cases the degree of inhibition was found to be dependent upon the concentration of sugar present. It was evident that several sugars, in concentrations at which they had no significant effect on the activity of toluene-treated cells, had a severely inhibitory effect on the activity of intact-cell preparations. The concentrations given in the table are the highest at which little or no inhibition of the activity of toluene-treated cells was observed.

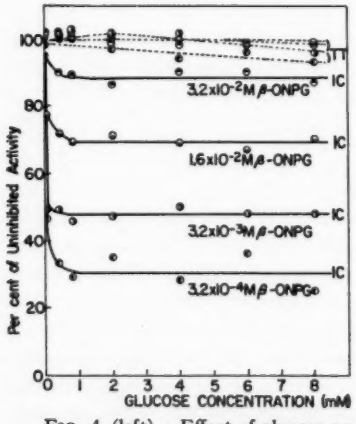
It was found that glucose, at concentrations of the order of 10^{-4} *M*, reduced the activity of intact *E. coli* B cells to a plateau level whereas, with the toluene-

TABLE I

Effect of sugars on the β-galactosidase activity of induced *E. coli* B

Sugar	Concentration (M)	% of uninhibited β-galactosidase activity	
		Toluene-treated organisms	Intact organisms
D-Glucose	1×10^{-3}	102	47
D-Fructose	2×10^{-3}	103	40
D-Mannose	2×10^{-3}	103	44
D-Galactose	1×10^{-2}	93	57
D-Arabinose	2×10^{-3}	100	91
L-Arabinose	2×10^{-3}	100	94
Lactose	1×10^{-3}	104	60
Melibiose	1×10^{-3}	100	48
Sucrose	1×10^{-2}	95	93
Raffinose	1×10^{-2}	101	33

treated preparations, a 10-fold or greater increase in glucose concentration was required before an appreciable inhibition was observed (Fig. 4). At every substrate concentration tested, inhibition by glucose of the activity of intact-cell preparations increased in a hyperbolic fashion with sugar concentration to a maximum at approximately 10^{-4} M glucose. When a concentration of glucose (8×10^{-3} M), maximally inhibitory to the β-galactosidase activity at all concentrations of substrate, was present, the relationship between activity and substrate concentration was as shown in Fig. 5. Thus, in the presence as well as in the absence of glucose, activity increased with substrate concentration in a hyperbolic manner. It is clear from the parallelism of the two curves



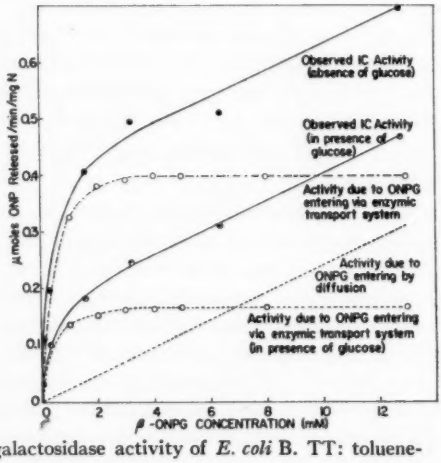

μ moles ONP Released/min/mg N
β-ONPG CONCENTRATION (mM)
Observed IC Activity (absence of glucose)
Observed IC Activity (in presence of glucose)
Activity due to ONPG entering via enzymic transport system
Activity due to ONPG entering by diffusion
Activity due to ONPG entering via enzymic transport system (in presence of glucose)

FIG. 4 (left). Effect of glucose on β-galactosidase activity of *E. coli* B. TT: toluene-treated cells. IC: intact cells.

FIG. 5 (right). Relationship between β-galactosidase activity of intact *E. coli* B and substrate concentration, in the presence and absence of glucose. TT: toluene-treated cells. IC: intact cells.

at concentrations of β-ONPG above 1.6×10^{-3} *M* that the diffusion process was unaffected by the presence of glucose. However, the activity due to the postulated enzymic transport system was seriously reduced.

Effect of Metabolic Inhibitors on β-Galactosidase Activity

The β-galactosidase activity of *E. coli* B in the presence of each of several metabolic inhibitors is shown in Table II. Fluoride and cyanide had no inhibitory effect on the activity of either intact cells or toluene-treated cell

TABLE II

Effect of metabolic inhibitors on β-galactosidase activity of induced *E. coli* B

Inhibitor*	Concentration (*M*)	% of uninhibited β-galactosidase activity	
		Toluene-treated organisms	Intact organisms
N-Ethylmaleimide	10^{-3}	101	34
Sodium iodoacetate	10^{-3}	101	37
Sodium fluoride	10^{-2}	100	100
Sodium cyanide	10^{-2}	93	140

*Inhibitor added 5 minutes prior to substrate.

preparations. However, at concentrations which had no inhibitory effect on the activity of toluene-treated cells the sulphydryl-complexing agents, N-ethylmaleimide and iodoacetate reduced the activity exhibited by intact cells to approximately 35% of the uninhibited value. As in the case of glucose, an increase in the concentration of N-ethylmaleimide reduced the activity to a minimum plateau level. This maximum degree of inhibition was not affected by the addition of glucose at a concentration (10^{-3} *M*), which by itself brought about approximately the same degree of inhibition of the β-galactosidase activity of intact-cell preparations.

Relationship between the β-Galactosidase Activities Exhibited by Intact Cell and by Toluene-treated Preparations

In Table III are given the data relating the β-galactosidase activity to the concentration of substrate at various levels of induction for intact cells (IC) and for toluene-treated preparations (TT). From these results it is evident that:

(i) while each of the toluene-treated preparations has reached maximum activity at a concentration of 3.2×10^{-3} *M* β-ONPG, only the non-induced (control) intact-cell preparation has done so even at a fourfold higher concentration (12.8×10^{-3} *M*).

(ii) the ratio of the activities (TT/IC) increases markedly as the degree of induction (and enzyme content) is increased. This observation is in agreement with that of Lederberg (2), who found that this ratio was dependent upon the β-galactosidase content of the cells.

TABLE III

TT/IC* activity ratios at different concentrations of substrate and different levels of β-galactosidase content

Concentration of β-ONPG ($M\times10^{-3}$)	A†			B			C		
	Enzyme activity		Ratio	Enzyme activity		Ratio	Enzyme activity		Ratio
	TT	IC	TT/IC	TT	IC	TT/IC	TT	IC	TT/IC
12.8	3.05	3.25	0.94	30.3	16.8	1.69	370	60.0	6.17
6.4	3.05	3.00	1.02	32.4	11.8	2.74	380	35.5	10.7
3.2	3.05	2.70	1.13	32.3	7.3	4.34	380	16.2	23.4
1.6	3.00	2.40	1.25	30.5	6.2	4.81	370	12.0	30.8
0.32	2.53	1.41	1.79	23.0	3.0	7.66	292	10.0	29.2
0.08	1.67	0.48	3.48	10.3	2.4	4.28	233	6.9	3.4

*TT: toluene-treated cells. IC: intact cells.
†A: cells induced 0 minutes and exhibiting TT β-galactosidase activity at 6.4 m*M* β-ONPG of 3.05.
B: cells induced 20 minutes and exhibiting TT β-galactoside activity at 6.4 m*M* β-ONPG of 32.4.
C: cells induced 90 minutes and exhibiting TT β-galactoside activity at 6.4 m*M* β-ONPG of 380.

Michaelis Constants for the β-Galactosidase Activity of E. coli B at Various Levels of Induction

The Michaelis constants for the β-galactosidase activity of toluene-treated preparations of *E. coli* B and the "apparent" constants for the activity of intact cells were determined with cells induced for various periods of time with 0.01 *M* lactose. The data in Table IV show that the K_M of β-galactosidase for β-ONPG, as measured in toluene-treated cells, was remarkably constant (1.7×10^{-4} *M*) over the entire 2000-fold range of enzyme levels. On the other hand, the constant, measured with intact cells, was different depending upon the β-galactosidase content of the cells. With organisms containing little enzyme the value of K_M for intact cells was similar to that of toluene-treated cells; with those containing large amounts of β-galactosidase, the constant was much lower.

TABLE IV

Measured Michaelis constants for the β-galactosidase activity of *E. coli* B induced to contain varying amounts of enzyme

Duration of induction (min)	β-galactosidase content (TT*activity at 3.2×10^{-3} *M* β-ONPG)	Measured K_M ($M\times10^{-4}$ β-ONPG)	
		IC*	TT*
0	0.024	1.7	1.6
10	0.46	1.7	1.4
20	6.66	0.21	1.7
90	35.2	0.33	1.4

*TT: toluene-treated cells. IC: intact cells.

Discussion

In 1950 Lederberg (2) suggested that in *E. coli* there is a permeability barrier limiting the rate of entrance of galactosidic substrates to the site of action of the enzyme, β-galactosidase. The data presented above support this conclusion. It seems clear that in *E. coli* B two distinct reactions mediate

the passage of β-ONPG into the cell. One appears to be a process of physical diffusion and the second to be catalyzed by an enzyme-like transport system. This conclusion was first indicated by the nature of the relationship between the β-galactosidase activity of *E. coli* B cells and the external concentration of β-ONPG. It was supported by the finding that the activity of intact cells could be only partially inhibited by sugars and by sulphydryl-complexing agents at concentrations such that these substances had no effect on β-galactosidase activity per se. In addition, it was found that the K_M for β-ONPG measured with intact cells was not constant in cells with varying β-galactosidase content. The data presented regarding the relationship between the β-galactosidase activity exhibited by toluene-treated preparations and that of intact *E. coli* B over a wide range of levels of induction are consistent with the above conclusions.

Direct evidence for a specific β-galactoside concentrating system (galactoside-permease) has been obtained by Rickenberg *et al.* (10, 11, 12). A similar mechanism which appears to concentrate both α- and β-galactosides was studied by Pardee (20). The relationship between the galactoside transport system described above and the galactoside-permease is not clear. Though in general their properties are similar, in certain respects they differ. The permease appears to be energy-dependent, since it is inhibited by 2,4-dinitrophenol and by azide and its action is accompanied by consumption of oxygen (21). In *E. coli* B the galactoside transport system appears not to be energy-dependent, since the β-galactosidase activity of intact cells is not inhibited by fluoride or cyanide. Cohen and Monod (22) have reported insensitivity of the β-galactosidase activity of intact *E. coli* ML to these two agents. In addition, the galactoside-permease is unaffected by the presence of glucose, whereas the galactoside transport system is severely inhibited by this sugar.

Acknowledgments

The authors are grateful for the generous financial assistance provided by the Advisory Committee on Scientific Research and the Advisory Committee on Medical Research of the University of Toronto and by the National Research Council of Canada. One (R.S.) is a Fellow of the National Cancer Institute of Canada.

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THE α-GALACTOSIDASE ACTIVITY OF ESCHERICHIA COLI B[1]

ROSE SHEININ[2] AND BRUCE F. CROCKER

Abstract

Evidence is presented that in intact *Escherichia coli* B the observed α-galactosidase activity is limited by one reaction under all conditions tested. It has therefore been concluded that such measurements of activity can be used to study the induced formation of this enzyme.

Introduction

The paper which follows (1) describes an investigation of the concurrent formation of α-galactosidase and β-galactosidase in *E. coli* B, stimulated by one of a number of inducers. To study the synthesis of these enzymes it must be possible to measure the enzyme content of the cells. The β-galactosidase content of *E. coli* B may be determined by using toluene-treated cells (2, 3, 4) or other preparations (5, 3, 6, 7). However, the α-galactosidase content of cells of *E. coli* B cannot be measured in such preparations. A study was therefore made of the α-galactosidase activity of intact cells of *E. coli* B to determine whether such activity represented a measure of the content of this enzyme in these organisms.

Materials and Methods

Organism and Its Cultivation

Escherichia coli B was maintained and cultivated according to procedures already described (4). Briefly, organisms were grown in a medium containing Tryptose (Difco), harvested in the early stationary phase of growth and resuspended in potassium phosphate buffer (0.05 *M*, pH 7.05). Growth was determined during the course of an experiment by measuring the optical density at 660 mμ in an Evelyn colorimeter. This wave length was chosen to eliminate the effect of a progressively developing yellow color in the cultures.

Induction of α-Galactosidase Formation

The formation of α-galactosidase in washed organisms was induced according to procedures already described (4), except that, in this case, melibiose (Nutritional Biochemicals Corp.: 0.12 *M*) was used as inducer. Unless otherwise noted, induction was carried out for 2 hours at 37° C. Enzyme formation was halted with chloromycetin (Parke, Davis and Co., 20 μg/ml). The induced organisms were harvested by centrifugation, washed with and resuspended in sodium phosphate buffer (0.05 *M*, pH 7.05), and stored at 2° C pending enzyme assay.

[1]Manuscript received June 27, 1960.
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Measurement of α-Galactosidase Activity

α-Galactosidase activity was measured with suspensions of intact *E. coli* B cells of standard density (4) using the chromogenic substrate, α-ONPG,* which was synthesized by the method of Porter *et al.* (8). Unless otherwise noted, α-ONPG was used at a final concentration of 1.6 m*M*. The time course of the activity was linear for at least 1 hour. Enzyme activity was measured in triplicate after a single time interval, which was varied with the level of enzyme present, and is expressed as μmoles ONP released/hour/mg bacterial nitrogen, as determined by a standard micro-Kjeldahl method.

Sugars and Galactose Derivatives

The sugars listed in Table II were of the highest purity obtainable from either British Drug Houses or Nutritional Biochemicals Corporation. α-MG* and β-MG* were synthesized by the method of Dale and Hudson (9). β-PRG* was generously provided by Dr. R. Holmes. α-ONPG,* β-ONPG,* and β-TPG* were synthesized according to the method of Seidman and Link (10).

Results

Extraction of α-Galactosidase from E. coli B

All attempts to extract α-galactosidase from *E. coli* B by means of fat solvents or surface active agents, lysis with phage, grinding, autolysis, ultrasonic treatment, or homogenization in 0.25 *M* sucrose have met with failure. The presence of substrate or sulphydryl compounds during the course of these treatments did not yield active enzyme. Similar frustration was experienced by Porter *et al.* (8) and by Hogness and Beatty (11).

Characteristics of the α-Galactosidase of Intact E. coli B

In Figs. 1–3 are illustrated some of the properties of the α-galactosidase of *E. coli* B. That a single enzyme reaction is under study under all conditions was indicated by the following observations:

(i) the activity followed simple Michaelis and Menten (12) kinetics;

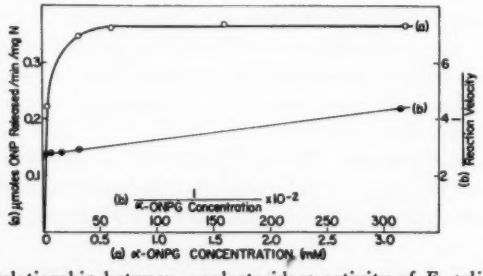

FIG. 1. (*a*) Relationship between α-galactosidase activity of *E. coli* B and substrate concentration. (*b*) Lineweaver–Burk plot of the above data.

*Abbreviations used: α-ONPG, *o*-nitrophenyl-α-D-galactoside; β-ONPG, *o*-nitrophenyl-β-D-galactoside; ONP, *o*-nitrophenol; α-MG, methyl-α-D-galactoside; β-MG, methyl-β-D-galactoside; β-PRG, propyl-β-D-galactoside; β-TPG, phenyl-β-D-thiogalactoside.

(ii) the relationship between enzyme activity and temperature over the range of 25° was described by a linear Arrhenius plot;

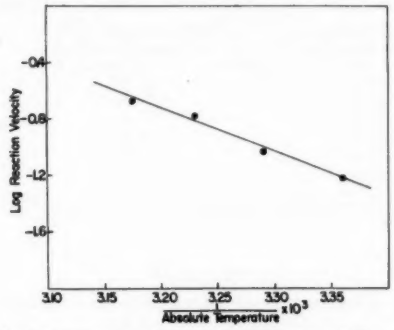

FIG. 2. Relationship between α-galactosidase activity of *E. coli* B and temperature (Arrhenius plot).

(iii) thermal inactivation of α-galactosidase activity at 54° C proceeded exponentially.

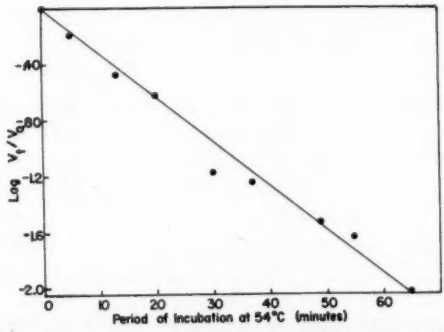

FIG. 3. Thermal inactivation of α-galactosidase activity of *E. coli* B. Organisms were equilibrated at 54° C for 5 minutes and then incubated at this temperature. Samples were removed at the times indicated to chilled centrifuge tubes, and their activities measured in the usual way. V_t, α-galactosidase activity at time t; V_0, enzyme activity at time 0, i.e. after equilibration.

Observed Michaelis Constants for the α-Galactosidase Activity of Intact E. coli B Induced to Contain Varying Amounts of Enzyme

Michaelis constants were determined for the α-galactosidase activity of *E. coli* B, induced for varying periods of time. In Table I it will be seen that, at all levels of enzyme activity, the K_M associated with this activity was the same, 5.5×10^{-5} *M* α-ONPG.

Effects of Sugars and Galactose Derivatives on α-Galactosidase Activity

The effects of a number of sugars and galactose derivatives on the α-galactosidase activity of intact *E. coli* B are shown in Table II. It was observed

TABLE I

Michaelis constants for α-galactosidase in *E. coli* B cells at various levels of induction

Expt. No.	Duration of induction (hr)	α-Galactosidase activity at 1.6 m*M* α-ONPG	Michaelis contant ($M \times 10^{-5}$ α-ONPG)
1	0.5	0.48	5.3
	2.0	2.36	5.7
	3.0	2.40	6.1
2	2.0	1.50	5.5
3	2.0	1.82	5.5
4	2.0	1.73	5.4

NOTE: *E. coli* B cells, grown and prepared as described in Methods, were induced for varying periods of time in the presence of 0.12 *M* melibiose.

TABLE II

Effect of presence of sugar analogues on the α-galactosidase activity of *E. coli* B

Inhibitor*	Final conc. ($M \times 10^{-3}$)	α-Galactosidase activity (% of uninhibited activity)
D-Arabinose	2	100
L-Arabinose	2	100
D-Xylose	2	96
D-Mannose	2	97
D-Fructose	2	94
D-Glucose	2	97
D-Galactose	10	56
α-MG	10	48
Melibiose	10	59
Raffinose	10	22
β-MG	10	80
β-PRG	10	80
Lactose	10	89
β-ONPG	10	104

*The inhibitor was dissolved in the substrate solution.

that, with the exception of galactose itself, the simple sugars had no significant effect on α-galactosidase activity. This observation differed markedly from that made for the β-galactosidase activity of *E. coli* B (4). All the galactosides except β-ONPG were inhibitory to α-galactosidase activity but it was noted that the α-galactosides produced much more marked inhibition than did the β-galactosides.

Effects of Enzyme Inhibitors on α-Galactosidase Activity

The effects of known enzyme inhibitors on the α-galactosidase activity of intact *E. coli* B are shown in Table III. The test compounds were added to suspensions of induced organisms 5 minutes prior to the addition of substrate. The pronounced inhibitory effect exerted by the sulphydryl-complexing agents, N-ethylmaleimide and iodoacetic acid, contrasts itself with that obtained with azide, cyanide, and fluoride which, as Aubel *et al.* (13) have shown, inhibit energy metabolism in *E. coli* cells. The action of N-ethylmaleimide and of

TABLE III

The effects of enzyme inhibitors on the activity of α-galactosidase in *E. coli* B

Inhibitor*	Final concentration ($M \times 10^{-3}$)	α-Galactosidase activity (% of uninhibited activity)
Sodium fluoride	1	95.0
	10	85.6
Sodium cyanide	1	85.6
	10	54.6
Sodium azide	1	70.5
Glutathione	1	100.
N-Ethylmaleimide	0.2	40.1
	1	15.5
N-Ethylmaleimide	1	
+ glutathione	1	103.
Iodoacetate	1	4.1
Iodoacetate	1	
+ glutathione	1	41.0

*The inhibitor was added to the cells 5 minutes before the substrate.

iodoacetate was totally or partially prevented by glutathione. These observations reflect a dependence of the α-galactosidase activity of *E. coli* B upon reduced sulphydryl groups, which are affected directly by N-ethylmaleimide and iodoacetate and indirectly by cyanide, etc., due to a general decrease of reducing power within the cell.

Deadaptation and α-Galactosidase Activity

When induced *E. coli* B was subsequently incubated in a growth medium without inducer, although the total activity of the bacterial population remained constant over the 2-hour period of the experiment, specific α-galactosidase activity of the cells decreased at a rate directly proportional to the increasing cell density (Fig. 4). Thus deadaptation resulted in a straightforward dilution of enzyme activity.

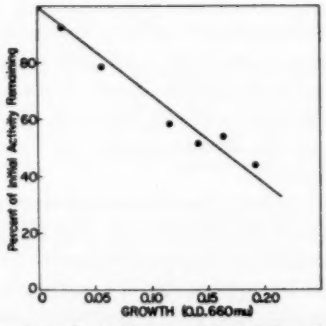

FIG. 4. Deadaptation and α-galactosidase activity of *E. coli* B. Induced cells of *E. coli* B were incubated at 37° C in a medium composed of buffer (sodium phosphate 0.05*M*, pH 7.05), yeast extract (Difco, 1.3% w/v) and sodium succinate (1.8% w/v).

Discussion

In the earliest studies of induced enzyme formation, investigators emphasized the need to establish that the observed increase in enzyme activity was due to the biosynthesis of enzyme protein and not to (*a*) an unmasking of precursor enzyme, (*b*) the formation of coenzymes or cofactors, or (*c*) a change in cell permeability allowing greater access of substrate to enzyme. Unmasking of active enzyme centers in precursor protein molecules by disruption of peptide bonds has been found to occur among proteolytic enzymes (14). Enzyme activation is known to result from the removal of a specific inhibitor or the appearance of a coenzyme or cofactor. However, none of these processes requires active protein synthesis and so may be excluded as an explanation of the observed increase in α-galactosidase activity of *E. coli* B which occurs in response to the presence of a number of galactose-containing compounds (1). In this case, the increase in activity may be prevented or brought to a halt by chloromycetin, a substance known to interfere with protein synthesis (15). In addition, the work of Hogness and Beatty (11), with enzyme extracted from *Aerobacter aerogenes*, gives no indication that α-galactosidase requires activation.

It is unlikely that the increase in α-galactosidase activity of *E. coli* B during induction is due to a non-specific change in cell permeability. The observed relationship between enzyme activity and the concentration of substrate indicated that diffusion plays no role in the α-galactosidase activity exhibited by this organism. Where diffusion does make a contribution to the enzyme activity of intact cells, as in the case of β-galactosidase activity of *E. coli* B (4) and of *E. coli* ML3 (16), this can readily be detected.

These investigations do not exclude the possibility that induction results in the formation of a specific galactoside transport system similar to the β-galactoside uptake mechanism described by Rickenberg *et al.* (17), Rotman (7, 18), and Holmes *et al.* (4). Indeed Pardee (19) has demonstrated the existence of an α-galactoside permeation system in *E. coli*. However, the data presented in the present paper indicate that in intact *E. coli* B a single enzyme reaction limits the observed α-galactosidase activity under all the conditions tested. The observed relationship between activity and substrate concentration, the constancy of the Michaelis constant at all levels of induction, the linear Arrhenius plot relating activity and temperature, the exponential inactivation at high temperature, and the simple dilution of enzyme activity during deadaptation, all testify to this.

Although the data reported force the conclusion that the α-galactosidase activity exhibited by *E. coli* B is limited by one reaction, they do not permit one to distinguish between the following two possibilities: (i) that the α-galactosidase is not separated by a permeability barrier from its substrate in the medium surrounding the cells and that the activity measured with intact cells reflects the amount of enzyme present in the cells, (ii) that the observed activity may be an indirect measure of the activity of an α-galactoside trans-

port system which, under all conditions, limits the amount of α-galactosidase activity observed. It would seem that the second of these possibilities is unlikely since this would require that the postulated transport system be limiting not only over a wide range of substrate concentrations but at all levels of induction, over a range of temperatures from 25° C to 40° C and during the entire time interval required for the thermal inactivation of the observed activity.

It will be possible to distinguish unequivocally between the above two hypotheses only when the α-galactosidase has been isolated from *E. coli* B and a comparative study has been made of the enzyme activity exhibited by intact cells and by cell-free extracts. For the purpose of the investigation referred to in the introduction and described in the following paper (1), it is not essential that this distinction be made. Whether α-galactosidase activity exhibited by intact *E. coli* B is, in fact, a direct measure of this enzyme or an indirect measure of an α-galactoside transport system, the determination of this activity does permit the determination of the activity of a reaction involved in the metabolism of α-galactoside. It is in this sense that the term "α-galactosidase activity" is used here and in the next communication.

Acknowledgments

The financial assistance of the Advisory Committee on Scientific Research and of the Advisory Committee on Medical Research of the University of Toronto as well as that of the National Research Council of Canada is gratefully acknowledged. One of the authors (R.S.) has been a Fellow of the National Cancer Institute of Canada.

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THE INDUCED CONCURRENT FORMATION OF α-GALACTOSIDASE AND β-GALACTOSIDASE IN ESCHERICHIA COLI B[1]

ROSE SHEININ[2] AND BRUCE F. CROCKER

Abstract

An investigation of the concurrent induction of α-galactosidase and β-galactosidase in *Escherichia coli* B has been made. It was observed that galactose and a number of α- and β-galactosides induced the biosynthesis of both enzymes. However, some α-galactosides stimulated the synthesis of only α-galactosidase and some β-galactosides induced the formation of only β-galactosidase. The significance of these observations in relation to the nature of the immediate inducer has been discussed.

Introduction

Pollock (1) and Cohn and Monod (2) have emphasized the wisdom of distinguishing, within the over-all process of induced enzyme formation, between (*a*) the synthesis of enzyme protein resulting from induction and (*b*) a metabolism of the inducer. It was suggested that, in this latter process, the externally applied inducer may enter the cell and, either during the process or subsequently, be converted to a different substance, the immediate inducer, which in turn enters into reaction with the enzyme-forming system. Information as to the nature of this immediate inducer would, of course, aid in an understanding of this critical induction reaction.

Although it has been known for some time that the α-galactoside, melibiose, can induce in *E. coli* the synthesis of β-galactosidase (3, 4, 5, 6, 7, 8) it was first demonstrated by Lester (9) that melibiose could stimulate simultaneously the formation of both α- and β-galactosidases. This finding was confirmed by Koppel *et al.* (10) and by Porter *et al.* (11), who also found that methyl-α-D-galactoside was similarly effective.

It was believed that an investigation of the concurrent formation of these two galactosidases, induced by single compounds, might provide clues as to the nature of the immediate inducer. The present communication describes such a study with galactose and eight derivatives (12). Since this investigation was completed, Hogness and Beatty (13) have reported the results of a similar study with *Aerobacter aerogenes*.

Materials and Methods

Organism and Cultivation

Escherichia coli B was maintained and cultivated as previously described (14). Briefly, organisms, grown to early stationary phase in tryptose medium,

[1]Manuscript received June 27, 1960.
Contribution from the Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada.
[2]Present address: Ontario Cancer Institute, 500 Sherbourne St., Toronto 5, Ontario.

were harvested, washed, and suspended in potassium phosphate buffer (0.05 *M*; pH 7) pending induction. Growth was determined during the course of an experiment by measuring the optical density at 660 mμ in an Evelyn colorimeter. This wave length was chosen to eliminate the effect of a progressively developing yellow color in the cultures.

Induction of Enzyme Formation

To induce formation of α-galactosidase, β-galactosidase, or both enzymes concurrently, washed organisms were incubated at 37° C with inducer, yeast extract, and sodium succinate in potassium phosphate buffer (0.05 *M*; pH 7) (14). Enzyme formation was halted by the addition of chloromycetin (Parke, Davis and Co.) to a final concentration of 20 μg/ml. Under the experimental conditions used, after an initial lag period, enzyme formation proceeded at a constant rate for approximately 80 minutes. The rate of enzyme formation was calculated from the linear portion of the plot relating enzyme activity to duration of induction. It is expressed in terms of enzyme activity formed/unit time/mg bacterial nitrogen, as determined by a standard micro-Kjeldahl method.

Measurement of Enzyme Activity

The α-galactosidase and β-galactosidase activities were determined by adaptations (14) of the method introduced by Lederberg (15), using the chromogenic substrates, α-ONPG* and β-ONPG* respectively. α-Galactosidase activity was measured with intact cells of *E. coli* B, using 1.6 m*M* α-ONPG (16); β-galactosidase activity was determined with toluene-treated cells, using 3.2 m*M* β-ONPG (14). Enzyme activity is expressed as μmoles ONP* released/unit time/mg bacterial nitrogen. Chloromycetin (20 μg/ml) was present during all determinations of enzyme activity.

Galactose and Its Derivatives

Galactose, free of glucose, was generously supplied by Dr. D. L. Michener. Melibiose and raffinose were obtained from Nutritional Biochemicals Corporation and lactose (Analar grade) from British Drug Houses. β-PRG* was prepared by the method of Holmes (17), who provided much of the material used. α-MG* was synthesized using the procedure of Dale and Hudson (18); β-MG* by that of Helferich and Schafer (19); α-ONPG by that of Porter *et al.* (11); β-ONPG and β-TPG* by that of Seidman and Link (20); α-PG* and β-PG* was prepared by F. M. Kahan according to the method of Helferich and Schafer (19).

Results

The Induced Biosynthesis of α-Galactosidase and β-Galactosidase

The results of a study of the formation of α-galactosidase and β-galacto-

*Abbreviations used: α-ONPG, *o*-nitrophenyl-α-D-galactoside; β-ONPG, *o*-nitrophenyl-β-D-galactoside; ONP, *o*-nitrophenol; β-PRG, propyl-β-D-galactoside; α-PG, phenyl-α-D-galactoside; β PG, phenyl-β-D-galactoside; α-MG, methyl-α-D-galactoside; β-MG, methyl-β-D-galactoside; β-TPG, phenyl-β-D-thiogalactoside; β-TMG, methyl-β-D-thiogalactoside.

sidase in *E. coli* B induced by galactose and some of its derivatives are summarized in Table I. With the exception of α-PG, inducers were tested over a

TABLE I

The capacity of various galactosides to induce the biosynthesis of α- and (or) β-galactosidase in *E. coli* B

	Inducer	Galactoside configuration	Aglycone	Induced synthesis of galactosidase α-	β-
(*a*)	Galactose	α, β	Hydrogen	+	+
	α-MG	α	Methyl-	+	+
	β-MG	β	Methyl-	+	+
	β-PRG	β	Propyl-	+	+
	Melibiose	α	Glucose	+	+
	Raffinose	α	Sucrose	+	+
(*b*)	Lactose	β	Glucose	−	+
	β-ONPG	β	*o*-Nitrophenyl-	−	+
(*c*)	α-ONPG	α	*o*-Nitrophenyl-	+	−
	α-PG*	α	Phenyl-	+	−

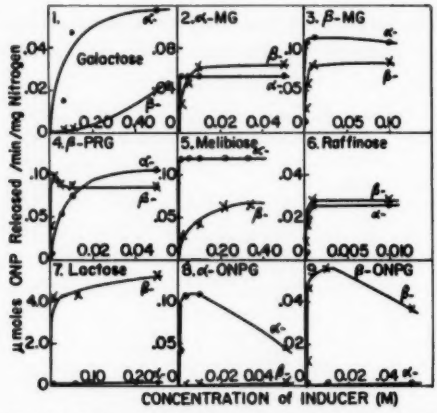
*The experiments with α-PG were preliminary in nature and indicated qualitatively that it stimulates the synthesis of only the α-galactosidase.

range of concentrations from 10^{-5} to 10^{-1} *M*; α-PG was used at 10^{-2} *M* only. The data confirm and extend previous observations. It is evident that the inducers tested fall into three groups. In the first group, consisting of those substances which induce the formation of both enzymes concurrently, are to be found galactose, aliphatic α- and β-galactosides, melibiose, and raffinose. In the second group, those substances which stimulate the formation of only α-galactosidase, are found the aryl α-galactosides, α-ONPG, and α-PG. Among those compounds which elicit the synthesis of only β-galactosidase are the β-galactosides, lactose, and the aryl derivative, β-ONPG. To this last group may be added β-TMG, studied by Sheinin and McQuillen (21).

The Relationship between Inducer Concentration and Rate of Induced Enzyme Formation

In Figs. 1–9 inclusive are illustrated the results obtained in studies of the response of *E. coli* B to induction by nine compounds, over a range of inducer concentrations. Under the experimental conditions employed, addition of inducer to the basal medium had no effect on the growth rate. Thus it would seem that the presence of these compounds had no significant effect on general protein synthesis but did specifically influence the formation of the galactosidases.

With most of the inducers the rate of enzyme formation increased with inducer concentration in a hyperbolic manner to reach a maximum. A decrease in rate at still higher concentrations of inducer was observed with the aryl galactosides, α-ONPG, and β-ONPG; this may reflect an inhibition of enzyme formation by the ONP released by galactosidase action. In the case of galactose increase of inducer concentration produced only a gradually increasing rate of β-galactosidase synthesis.



FIGS. 1–9. Relationship between the rate of induced formation of α- or β-galactosidase and inducer concentration. To obtain the experimentally observed values for the rates of β-galactosidase formation the values given in Fig. 2 must be multiplied by 10 and those in Figs. 1, 3, 4, and 5 by 100.

Relative Rates of Formation of α-Galactosidase and β-Galactosidase

The relative rates of formation of the galactosidase enzymes (calculated as indicated under Materials and Methods) elicited by the different inducers was examined, using concentrations of each which stimulated maximum rates of enzyme formation. This concentration had been assessed in the foregoing experiments. The observations presented in Table II indicate that the ratios

TABLE II

Rates of induced synthesis of α- and β-galactosidase at the optimum concentration of inducer

Inducer	Final concn. (*M*)	Rate of galactosidase synthesis α-	Rate of galactosidase synthesis β-	Ratio of rates (β/α)
(*a*) Galactose	0.50	7.1	321	46
α-MG	0.06	14.2	272	19
β-MG	0.01	12.9	728	56
β-PRG	0.06	7.3	667	86
Melibiose	0.22	11.7	459	39
Raffinose	0.001	6.3	111	18
(*b*) Lactose	0.25	0	438	∞
β-ONPG	0.01	0	4	∞
(*c*) α-ONPG	0.01	5.8	0	0

NOTE: *E. coli* B cells were induced with the compounds and at the concentrations shown in the table. Time progress curves were determined and the rates of enzyme formation calculated from the linear portions of these curves. The rate of enzyme synthesis is expressed as the increase in the units of enzyme (μmoles ONP/hr/mg/N) per minute of induction. The figures given are the observed rates × 10^3.

of the rates of formation of β-galactosidase and α-galactosidase varied between the extremes of ∞ and 0 (with those compounds stimulating the synthesis of only one of the two enzymes) and within a narrower range of 18 to 86 (with compounds inducing the concurrent formation of both enzymes).

Inhibition of Induced Enzyme Formation

An attempt was made to dissociate two distinct pathways of induction, one for α-galactosidase and one for β-galactosidase, by means of inhibitors. Monod *et al.* (3) had observed that β-TPG was a strong inhibitor of the induced synthesis of β-galactosidase in *E. coli* ML. The effects of the presence of this compound on the induced formation of each of the two galactosidases in *E. coli* B was investigated with the result illustrated in Fig. 10. It should be

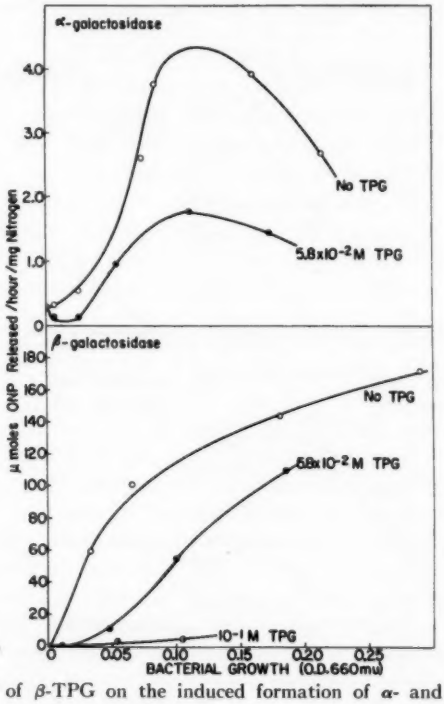


FIG. 10. Effect of β-TPG on the induced formation of α- and β-galactosidase in *E. coli* B.

noted that the specific inducers, α-ONPG and lactose, were used in order to obviate the possible interactions of concurrent synthesis of the two enzymes. It will be seen that β-TPG inhibits the formation of α-galactosidase induced by an α-galactoside as well as the formation of β-galactosidase induced by a β-galactoside.

Glucose, which had been demonstrated previously (22, 23) to be an inhibitor of the induced formation of β-galactosidase and of other enzymes, inhibited the induction of α-galactosidase by α-ONPG and of β-galactosidase by lactose. Furthermore, it was observed that inhibition of the induced synthesis of both α- and β-galactosidases occurred in the presence of the dual

inducer, melibiose. In each case, i.e. with β-TPG or glucose, addition of inhibitor had little or no effect on the growth of the organisms.

Interactions between Inducers

Interesting examples of interaction between inducers in *E. coli* B are shown in Fig. 11. Lactose, which itself stimulated the synthesis of only β-galactosidase, completely inhibited the formation of α-galactosidase normally induced by raffinose. On the other hand, the presence of raffinose did not significantly depress the formation of β-galactosidase induced by lactose. In

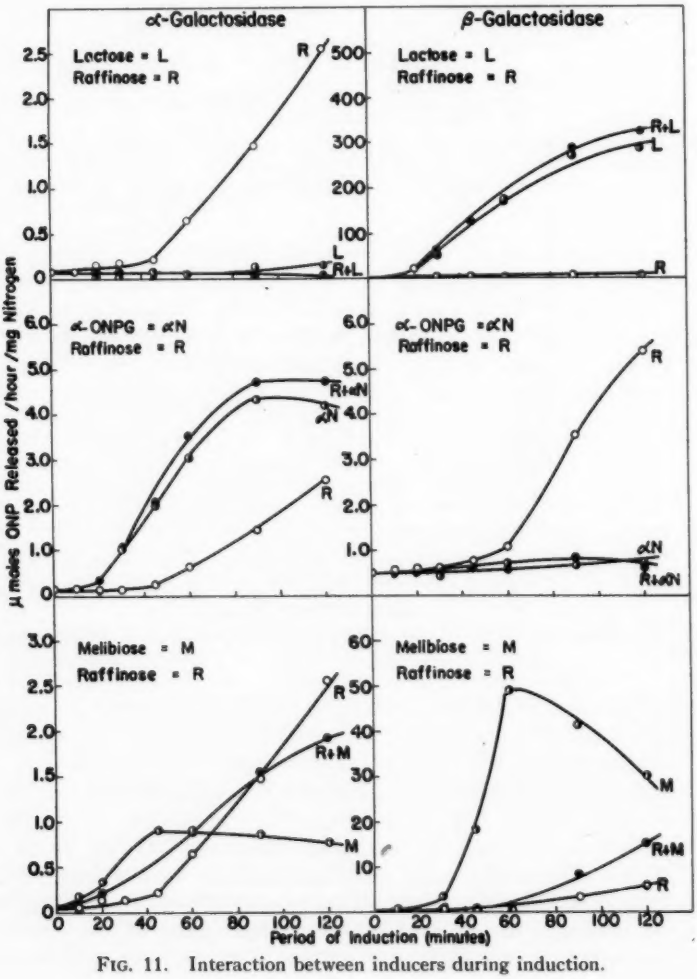


FIG. 11. Interaction between inducers during induction.

a similar manner, α-ONPG, which itself stimulated the formation of only α-galactosidase, completely inhibited the synthesis of β-galactosidase normally induced by raffinose. In the presence of both α-ONPG and raffinose, formation of α-galactosidase was, if anything, slightly greater than when α-ONPG alone was present. With melibiose, which induces the formation of both enzymes, the result was not as clear cut. The synthesis elicited in the presence of melibiose and raffinose together is intermediate between that obtained with either alone.

Discussion

Consideration of the possibilities as to the nature of the actual or immediate inducer involved in the formation of α-galactosidase and β-galactosidase in *E. coli* B indicated that there were at least four such types of compound:

(i) A-galactoside, i.e. the original aglycone, A, is still bound and the immediate inducer is identical with the external inducer. Galactose would be a special case in that no aglycone is involved.

(ii) Galactose, i.e. the aglycone group has been removed and the immediate inducer produced from all externally applied galactosides is, in fact, galactose itself.

(iii) X-galactoside, i.e. the original aglycone, A, has been replaced by a new aglycone, X, supplied by the cell. This would represent a transgalactosidation reaction.

(iv) A-galactoside-Y, i.e. a new group, Y, has been added, leaving intact the original galactosidyl linkage.

The second and third postulates, each of which implies that a common immediate inducer is produced from whatever galactoside may be present in the external medium, are inadequate on two counts: (*a*) Monod *et al.* (3, 24) have demonstrated that melibiose and β-TMG, which do not support the growth of *E. coli* ML and are not hydrolyzed by β-galactosidase, are potent inducers of this enzyme. Induction of enzyme formation by non-utilizable inducers has also been observed by Pardee (8) and by Sheinin and McQuillen (21); (*b*) on the basis of either postulate it would be expected that any compound which can act as inducer of one galactosidase would also act as inducer of the second galactosidase. This is manifestly not borne out by experiment. Further, and on the same basis, in cases where formation of both enzymes is induced one would expect the ratio of the two rates to be constant from inducer to inducer. This is definitely not the case experimentally.

The possibility that α-galactosidyl compounds induce the formation of β-galactosidase by first inducing the synthesis of α-galactosidase and liberating galactose (and conversely for β-galactosidyl compounds) appears to be ruled out on the ground that if such a phenomenon were involved with inducers of both enzymes, one would expect all the galactosides tested to induce formation of both galactosidases since all are hydrolyzable by the homologous enzyme.

It might be argued that the aglycones, released by hydrolysis of some

galactosides, are inhibitory to formation of one of the two enzymes and not to that of the other. However, the exclusive induction of the homologous galactosidase by various non-metabolizable inducers is not consistent with this hypothesis. Further, in the case of melibiose studied by Monod *et al.* (3) in *E. coli* ML, an α-galactoside, which cannot be hydrolyzed by this organism, induces the formation of β-galactosidase. And again, the presence of glucose is clearly inhibitory to the induced formation of both α- and β-galactosidase in *E. coli* B. At low concentration (10^{-5} *M*) Porter *et al.* (11) observed stimulation by glucose of the induced formation of β-galactosidase. Thus, if released (from lactose for example) in small quantity, glucose should stimulate enzyme formation, and, if released in large amount, it should inhibit the formation of both enzymes. It would seem unlikely, therefore, that specific inhibition of heterologous enzyme formation by a split product of the inducer can explain the induction by some galactosides of the formation of only homologous enzyme.

That the same immediate inducer, common to all externally applied inducers, is not involved was also indicated by Landman (25), who observed that, when two galactosides were present simultaneously, their inductive effects were not additive. They reasoned that if the same inducer were formed from each of the two galactosides, the effects should have been additive.

On the basis of the evidence discussed, it has therefore been concluded that the immediate inducer of β-galactosidase in *E. coli* is neither the product of a hydrolytic nor of a transgalactosidation reaction formed from all externally applied inducers. Thus it would seem reasonable to conclude that the original galactosidic bond is present in the immediate inducer. This conclusion bears the corollary that each externally applied inducer gives rise to a different and specific immediate inducer, all of which have, in common, a galactosidyl group.

Either hypothesis (i) or (iv), which postulate respectively that the immediate inducer is the same as that applied externally or is a modified form of the latter, would be consistent with the available evidence. Pardee (8), Rickenberg *et al.* (26), and Sistrom (27) have demonstrated that melibiose, lactose, and β-TMG can be recovered in unchanged form from *E. coli* after uptake by a galactoside- "permease" (26). In a cryptic mutant of *E. coli* ML, Herzenberg (28) found that β-ONPG enters the cells solely by diffusion. Since these organisms can be induced to form β-galactosidase, the immediate inducer could be unaltered, simply diffused, material. Although these observations show that inducer inside and outside the permeability barrier may exist in the same form, they do not exclude a further step in inducer metabolism to give rise to an immediate inducer of the form postulated in hypothesis (iv).

In the present studies a number of α-galactosides were found to stimulate only α-galactosidase formation and some β-galactosides to induce only β-galactosidase synthesis. Similar results were obtained by Hogness and Beatty (13). In accord with current concepts of the specificity of protein

synthesis, it might be postulated that there are two distinct enzyme-forming systems and that each reacts only with inducer of the homologous configuration. The latter part of this hypothesis is invalidated by the observations of Monod *et al.* (3) and of Pardee (8) that the α-galactoside, melibiose, which is neither metabolized nor hydrolyzed by *E. coli* ML, nevertheless induced in these organisms the synthesis of β-galactosidase. Rather it would seem most reasonable to conclude that both α- and β-galactosides may interact with the α- and β-enzyme-forming systems. The exclusive synthesis of one enzyme by non-hydrolyzable inducers could be explained in terms of steric hindrance of reaction with the heterologous enzyme-forming site, while the more general concurrent synthesis of the two galactosidases at different relative rates would reflect differences in the relative affinities of the individual inducers for the two enzyme-forming systems.

In explanation of the action of inducers, which are hydrolyzable by the induced galactosidase and yet induce exclusively the formation of only one of the two galactosidases, it should be pointed out that galactose, at least in *E. coli* B, is a relatively poor inducer requiring concentrations 50- to 100-fold that of galactoside inducers to stimulate enzyme synthesis at comparable rates. Thus it would seem that the galactose moiety, the aglycone, and the configuration about C_1 would all play a part in determining the relative affinity of the immediate inducer for the two enzyme-forming sites and, therefore, the rate and extent of the formation of the two galactosidase enzymes.

Acknowledgments

The authors are grateful for the generous financial assistance afforded by the Advisory Committee on Scientific Research and the Advisory Committee on Medical Research of the University of Toronto as well as that of the National Research Council of Canada and of the National Cancer Institute of Canada.

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THE EFFECT OF RIBOFLAVIN DEFICIENCY AND GALACTOFLAVIN FEEDING ON OXIDATIVE PHOSPHORYLATION AND RELATED REACTIONS IN RAT LIVER MITOCHONDRIA[1]

ROBERT E. BEYER,[2] STANLEY L. LAMBERG, AND M. ARTHUR NEYMAN[3]

Abstract

The effect of riboflavin deficiency and feeding of galactoflavin on the flavin content of liver mitochondria, oxidative phosphorylation, P_i–ATP exchange, and ATPase activity was studied. Both dietary riboflavin deprivation and galactoflavin feeding resulted in a depressed flavin content of mitochondria, the latter treatment resulting in a more severe flavin loss. P/O ratios under all treatments were normal as were P_i–ATP exchange rates and the oxidation of succinate. Glutamate and β-hydroxybutyrate oxidations were depressed in mitochondria from rats fed galactoflavin for 15 or 28 days. DNP-activated ATPase was elevated in both flavin-deficient and galactoflavin-fed preparations while Mg^{++}-activated ATPase was depressed in the galactoflavin-fed preparations. These results are discussed in relation to the hypothesis that flavin is involved in energy conservation in the diaphorase region of electron transport.

Introduction

Lindberg *et al.* (2) have postulated recently that diaphorase-flavin of the mitochondrial respiratory chain may be involved in the initial reactions involving the synthesis of ATP[4] from ADP and P_i. This suggestion was based upon (*a*) indications that mitochondrial P_i–ATP exchange and DNP-activated ATPase reactions involved a reversible electron transport through the diaphorase-FAD (3, 4) as well as (*b*) quantum-chemical considerations (5, 6). Evidence supporting the thesis that the P_i–ATP exchange reaction and DNP-activated ATPase are related solely to the phosphorylative step between DPN and cytochrome *b* has also appeared from other laboratories (7, 8). Löw (9, 10) and Löw *et al.* (11) have interpreted recent findings as providing evidence in support of such a postulated flavin involvement in the conservation of energy during the passage of electrons in the span DPNH → cytochrome *b*. The experiments reported here were undertaken in an attempt to provide further information concerning flavin involvement in oxidative phosphorylation and related reactions.

[1]Manuscript received July 12, 1960.

Contribution from the Department of Physiology, Tufts University School of Medicine, Boston, Mass. This work was done under the terms of Contract AT(30-1)911 of the Department of Physiology, Tufts University School of Medicine, with the U.S. Atomic Energy Commission. Portions of this work were presented (1) before the American Society of Biological Chemists, Chicago, Illinois, April, 1960.

[2]Senior Research Fellow of the U.S. Public Health Service.

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[4]The following abbreviations are employed: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenylic acid; P_i, inorganic orthophosphate; DNP, 2,4-dinitrophenol; FAD, flavin adeninedinucleotide; FMN, flavin mononucleotide; DPN, diphosphopyridinenucleotide; DPNH, reduced diphosphopyridinenucleotide; TPNH, reduced triphosphopyridinenucleotide; DOC, deoxycholate; Tris, trishydroxymethylaminomethane; B-OH, β-hydroxybutyrate; Mg eq., mitochondria derived from a given weight, in milligrams, of fresh tissue; P/O, microatoms of phosphorus esterified divided by microatoms of oxygen consumed.

Experimental

Weanling albino rats of the Wistar strain were placed on either a vitamin B complex test diet or a diet deficient in riboflavin[5] for varying periods of time. Adult riboflavin-deficient rats (ca. 200–250 grams) were obtained by feeding the riboflavin-deficient diet to which galactoflavin,[6] a flavin inhibitor, had been added (1 mg per gram) (12). Animals receiving the riboflavin-deficient, galactoflavin-enriched diet for approximately three weeks were employed in the experiments.

Animals were sacrificed by decapitation, the livers were removed rapidly and weighed, and twice-washed mitochondria were prepared essentially according to Schneider and Hogeboom (13) in 0.25 *M* sucrose. Mitochondrial nitrogen was determined by the Kjeldahl procedure upon aliquots of the mitochondrial suspensions following treatment with sonic vibration to obtain uniform suspensions. Oxygen consumption was measured by the conventional Warburg manometric technique at 30° C with air as the gas phase. Phosphate esterification was determined according to the isotopic distribution method of Lindberg and Ernster (14). The P_i–ATP exchange reaction was measured according to Löw *et al.* (4) and ATPase activities according to Siekevitz *et al.* (3). Total mitochondrial flavin content was measured spectroscopically according to Mahler (15) using FAD as reducible standard and dithionite as the reducing agent.

Results

Prior to performing experiments on mitochondrial preparations from riboflavin-deficient animals, the flavin content and oxidative phosphorylation with glutamate as substrate were studied employing mitochondria derived from fasted rats. Since it is known that animals on riboflavin-deficient diets consume less food and gain weight less rapidly than do animals on complete diets, it was thought advisable to determine the necessity of including pair-fed controls in our experiments. The data in Table I indicate that adult rats fasted for 4 or 7 days, but allowed water ad libitum, contained normal liver mitochondrial flavin levels on a mg N basis, and that such mitochondrial preparations exhibited no defect in the ability to esterify inorganic phosphate. Therefore, pair-fed controls have not been employed.

The effect of dietary riboflavin deprivation for various periods of time on liver mitochondrial flavin contents is recorded in Table II. It appears that

[5]Obtained from Nutritional Biochemicals Corp. The composition of the riboflavin-deficient diet is as follows: 18% "vitamin-free" casein, 68% sucrose, 10% vegetable oil, 4% U.S.P. salt mixture No. 2, and a vitamin fortification mixture lacking riboflavin. The vitamin fortification mixture contained the following in g/100 lb diet: vitamin A concentrate (200,000 units/g), 4.5; vitamin D concentrate (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; pyridoxine hydrochloride, 1.0; thiamine hydrochloride, 1.0; calcium pantothenate, 3.0. The vitamin fortification mixture also contained in mg/100 lb diet: biotin, 20; folic acid, 90; vitamin B_{12}, 1.35. The complete diet consisted of the above riboflavin-deficient diet with the addition of 1 g of riboflavin per 100 lb of diet.

[6]Kindly supplied by Dr. Karl Folkers, Merck Sharpe and Dohme Co.

TABLE I

Effect of fasting on flavin content and P/O ratio of liver mitochondria

(Each Warburg vessel contained mitochondria, 200 mg eq.; KCl, 150 μmoles; P_i, 50 μmoles; AMP, 4.3 μmoles; glucose, 60 μmoles; sucrose, 125 μmoles; potassium glutamate, 30 μmoles; $MgCl_2$, 7.5 μmoles; hexokinase, 330 KM units; reaction volume, 2 ml; temperature, 30° C; time of incubation, 20 minutes. Each value represents the mean of six measurements and is followed by the standard error of the mean in parentheses)

Period of fast, days		Flavin/1000 mg eq., mμmoles		Flavin/mg N, mμmoles		P/O	
Control	Exptl.	Control	Exptl.	Control	Exptl.	Control	Exptl.
0	4	21.2(0.51)	37.0(1.64)	12.2(0.57)	12.7(0.86)	2.84(0.04)	2.80(0.06)
0	4	19.6(1.00)	35.4(1.04)	11.6(0.72)	11.9(0.59)	2.89(0.05)	2.78(0.08)
0	7	28.8(1.26)	29.6(1.21)	11.8(0.63)	12.9(0.69)	2.72(0.04)	2.69(0.05)
0	7	26.4(1.12)	34.4(1.37)	11.6(0.69)	12.6(0.81)	2.81(0.04)	2.76(0.05)

TABLE II

Effect of riboflavin deprivation on flavin contents of isolated mitochondria
(Values in parentheses refer to the standard error of the mean,
the mean values being derived from six measurements)

Exptl. series[a]	Weeks on diet	Flavin/1000 mg eq., mμmoles		Flavin/mg N, mμmoles	
		Control	Experimental	Control	Experimental
1	1	14.7*(0.55)	8.4*(0.21)	5.35*(0.25)	3.08*(0.32)
2	2	18.5*(0.72)	9.7*(0.53)	5.95*(0.46)	3.22*(0.57)
3	3	20.6*(0.96)	8.8*(0.47)	6.08*(0.36)	2.49*(0.43)
4	4	21.4*(0.49)	9.7*(0.76)	5.28*(0.42)	2.30*(0.40)
5	5	15.1*(0.77)	6.3*(0.82)	5.92*(0.61)	1.65*(0.28)
6	6	15.1*(0.59)	7.6*(0.64)	5.61*(0.51)	2.54*(0.41)
7	12	24.8*(1.21)	9.8*(0.57)	7.61*(0.37)	2.19*(0.37)
8	14	18.9*(0.82)	8.0*(0.62)	5.76*(0.44)	1.90*(0.66)

[a]Series numbers correspond to those in Tables III, IV, and V.
*Indicates a statistically significant difference between control and experimental values. $P = 0.01$ or less.

deprivation periods as short as 1 week were sufficient to lower the reducible flavin content to about one-half the control level when calculated on a milligram equivalent or a milligram nitrogen basis. Following 1 week on the riboflavin-deficient diet, the loss of reducible flavin is extremely slow, the lowest level being approximately two millimicromoles per milligram nitrogen or about 30% of the control values. Maintaining animals on the deficient diet for as long as 3.5 months does not appear to result in a further flavin decrease. Mitochondrial suspensions from animals of the experimental series noted in Table II were assayed for the ability to carry out oxidative phosphorylation with succinate or glutamate as substrates. No consistent differences were observed between experimental and control groups despite the extended periods of riboflavin deprivation employed in series 7 and 8 (Table III). The addition of cytochrome *c* and FAD to the flasks containing succinate, and of DPN, cytochrome *c*, and FAD to flasks containing glutamate, did not result in experimental differences.

Mitochondrial ATPases were also studied in preparations of the same experimental series utilized in Table III, and the data resulting from such experiments are recorded in Table IV. Since two pathways appear to exist for the enzymic hydrolytic degradation of ATP in mitochondria (3, 16), one of which is stimulated by DNP and the other by magnesium following physical alteration of the particle (3), both pathways have been studied. In none of the experimental series do the resting ATPase levels (no flask additions) differ significantly as to control and experimental values. The addition of FAD to such resting mitochondria in most cases appeared to cause some slight stimulation, but these differences were not significant and no reliable trend was noted. Dinitrophenol resulted in a stimulation of ATPase activity in all cases and a statistically significant trend appeared beginning with the sixth week of riboflavin deprivation and lasting until the end of the 14-week experimental period. The addition of FAD to the DNP-activated ATPase did not

TABLE III

Effect of riboflavin deprivation on oxidative phosphorylation

(Experimental details as in Table I. Substrate, 30 μmoles/flask; DPN, 5×10^{-4} *M*, final concn.; cyt. *c*, 8.3×10^{-6} *M*, final concn.; FAD, 5×10^{-6} *M*, final concn. All values represent means from six experimental observations. Standard error in parentheses).

Exptl. series[a]	Weeks on diet	Substrate	Flask additions	O_2 consump., μatoms/mg N/hr		P/O	
				Control	Exptl.	Control	Exptl.
1	1	Succinate	None	55.06(2.1)	55.17(2.6)	1.81(0.07)	1.86(0.04)
			Cyt. *c*, FAD	54.97(1.9)	54.92(2.7)	1.37(0.10)	1.52(0.08)
		Glutamate	None	60.11(2.2)	55.31(3.1)	2.44(0.09)	2.50(0.07)
			DPN, cyt. *c*, FAD	48.58(2.9)	54.52(2.2)	1.37(0.17)	1.90(0.11)
2	2	Succinate	None	52.89(2.7)	63.20(2.4)	1.83(0.06)	1.86(0.09)
			Cyt. *c*, FAD	57.32(2.6)	70.44(3.4)	1.58(0.12)	1.87(0.13)
		Glutamate	None	61.85(1.6)	62.24(2.1)	2.56(0.11)	2.64(0.09)
			DPN, cyt. *c*, FAD	66.98(3.0)	66.72(2.7)	2.63(0.09)	2.45(0.11)
3	3	Succinate	None	45.06(2.1)	50.82(2.9)	1.71(0.04)	1.70(0.07)
			Cyt. *c*, FAD	50.07(2.8)	51.84(1.8)	1.50(0.09)	1.75(0.14)
		Glutamate	None	61.24(1.9)	64.89(3.2)	2.33(0.10)	2.16(0.06)
			DPN, cyt. *c*, FAD	71.04(2.4)	73.73(2.6)	2.06(0.08)	2.20(0.12)
4	4	Succinate	None	39.01(2.0)	39.69(2.6)	1.81(0.07)	1.76(0.09)
			Cyt. *c*, FAD	43.26(3.2)	37.05(2.5)	1.77(0.06)	1.82(0.11)
		Glutamate	None	53.91(2.3)	51.21(1.9)	2.24(0.04)	2.23(0.05)
			DPN, cyt. *c*, FAD	52.86(2.7)	58.47(3.1)	2.08(0.07)	1.96(0.09)
5	5	Succinate	None	48.49(3.6)	38.97(2.8)	1.51(0.11)	1.83(0.08)
			Cyt. *c*, FAD	58.03(3.2)	43.49(3.1)	1.59(0.06)	1.75(0.12)
		Glutamate	None	62.66(4.1)	40.45(3.4)	2.46(0.11)	2.51(0.09)
			DPN, cyt. *c*, FAD	70.12(4.2)	48.53(3.9)	2.33(0.06)	2.29(0.10)
6	6	Succinate	None	40.35(2.1)	46.68(2.6)	1.57(0.08)	1.64(0.13)
			Cyt. *c*, FAD	47.13(1.9)	48.41(2.3)	1.52(0.06)	1.63(0.09)
		Glutamate	None	37.01(3.4)	32.20(2.9)	2.26(0.04)	2.25(0.07)
			DPN, cyt. *c*, FAD	45.87(2.2)	42.33(2.3)	2.05(0.09)	2.23(0.11)
7	12	Succinate	None	43.80(4.1)	30.75(3.1)	1.79(0.05)	1.76(0.07)
			Cyt. *c*, FAD	47.25(3.2)	31.46(3.3)	1.81(0.09)	1.70(0.07)
		Glutamate	None	49.77(2.9)	40.65(2.6)	2.62(0.10)	2.69(0.09)
			DPN, cyt. *c*, FAD	52.55(3.8)	41.13(2.0)	2.25(0.11)	2.31(0.06)
8	14	Succinate	None	34.62(2.3)	37.41(2.5)	1.64(0.08)	1.55(0.10)
			Cyt. *c*, FAD	26.53(2.2)	38.47(3.7)	1.74(0.11)	1.61(0.06)
		Glutamate	None	35.51(2.8)	43.20(2.9)	2.58(0.05)	2.57(0.08)
			DPN, cyt. *c*, FAD	35.91(2.2)	46.86(3.2)	2.51(0.11)	2.55(0.09)

[a]Series numbers correspond to those in Tables II, IV, and V.

TABLE IV

The effect of riboflavin deprivation on mitochondrial ATPases
(Incubation was performed in 25-ml Erlenmeyer flasks shaken at 30° C for 20 minutes. Each flask contained 50 mg eq. rat liver mitochondria, 0.05 *M* Tris, 4 μmoles ATP, 0.25 *M* sucrose; when added, 0.1% DOC, 4 m*M* $MgCl_2$, 0.1 m*M* DNP, 5μ*M* FAD. Final volume, 2 ml. All values represent means of six experimental observations. Standard error in parentheses)

Exptl. series[a]	Weeks on diet	Flask additions	P_i liberated, μmoles/mg N/hr	
			Control	Experimental
1	1	None	13.91(1.1)	16.20(0.9)
		FAD	21.30(1.6)	15.99(1.1)
		DNP	34.56(1.2)	22.34(0.6)
		DNP, FAD	28.70(1.4)	17.96(0.9)
		Mg^{++}	8.50(0.4)	13.58(0.8)
		Mg^{++}, DOC	48.90(1.0)	36.57(1.1)
		Mg^{++}, DOC, FAD	26.96(0.8)	30.87(1.7)
2	2	None	13.08(1.2)	18.48(1.8)
		FAD	16.15(1.7)	18.68(1.6)
		DNP	26.92(1.4)	37.35(3.5)
		DNP, FAD	29.81(1.9)	24.24(2.1)
		Mg^{++}	18.08(1.6)	15.70(1.8)
		Mg^{++}, DOC	52.31(2.4)	71.52(3.6)
		Mg^{++}, DOC, FAD	57.89(3.3)	67.95(3.4)
3	3	None	8.50(1.4)	9.15(1.3)
		FAD	22.83(1.7)	16.27(1.6)
		DNP	30.27(1.6)	30.85(1.6)
		DNP, FAD	26.55(2.0)	33.39(1.4)
		Mg^{++}	12.21(1.2)	12.37(1.4)
		Mg^{++}, DOC	45.13(2.5)	44.41(2.5)
		Mg^{++}, DOC, FAD	54.51(2.1)	57.80(2.3)
4	4	None	7.56(1.5)	11.23(1.2)
		FAD	8.30(1.7)	12.65(1.3)
		DNP	27.56(2.0)	20.05(1.7)
		DNP, FAD	20.15(1.6)	19.91(1.6)
		Mg^{++}	11.56(1.8)	17.35(2.0)
		Mg^{++}, DOC	36.74(1.5)	40.38(2.1)
		Mg^{++}, DOC, FAD	34.37(2.3)	37.82(1.9)
5	5	None	9.41(1.4)	6.42(1.3)
		FAD	9.41(1.3)	7.05(1.6)
		DNP	32.00(2.2)	36.97(2.4)
		DNP, FAD	25.65(1.9)	30.71(2.2)
		Mg^{++}	40.71(2.0)	10.03(2.4)
		Mg^{++}, DOC	59.53(3.6)	44.49(2.2)
		Mg^{++}, DOC, FAD	47.76(2.1)	44.18(2.1)
6	6	None	9.59(1.9)	7.63(1.7)
		FAD	8.70(1.4)	8.83(1.3)
		DNP	26.77*(2.1)	44.95*(2.3)
		DNP, FAD	17.40*(1.9)	38.53*(2.1)
		Mg^{++}	15.17(2.3)	12.64(1.9)
		Mg^{++}, DOC	39.93(3.1)	46.16(3.2)
		Mg^{++}, DOC, FAD	46.39(2.9)	47.56(2.8)
7	12	None	9.02(2.0)	8.04(2.3)
		FAD	10.86(1.6)	11.64(1.9)
		DNP	32.58*(2.0)	50.49*(2.2)
		DNP, FAD	34.23*(2.2)	47.14*(1.8)
		Mg^{++}	14.72(1.9)	12.46(1.6)
		Mg^{++}, DOC	54.85(3.3)	58.13(2.7)
		Mg^{++}, DOC, FAD	64.05(3.4)	55.85(3.4)

TABLE IV (*Concluded*)

Exptl. series[a]	Weeks on diet	Flask additions	P_i liberated, μmoles/mg N/hr	
			Control	Experimental
8	14	None	15.00(2.4)	12.97(1.9)
		FAD	13.54(2.4)	13.54(2.2)
		DNP	28.35*(2.2)	56.44*(2.1)
		DNP, FAD	25.79*(2.4)	60.71*(2.3)
		Mg^{++}	22.32(1.9)	17.67(3.2)
		Mg^{++}, DOC	76.28(3.2)	64.28(3.6)
		Mg^{++}, DOC, FAD	66.40(2.9)	59.29(2.4)

[a]Series numbers correspond to those in Tables II, III, and IV.
*Indicates a statistically significant difference between control and experimental values. $P = 0.01$ or less.

obliterate the increased ATPase activity in the riboflavin-deficient preparations. Observations on the magnesium-activated ATPase revealed no trend such as that seen in the DNP-activated ATPase in the deficient preparations.

The mitochondrial P_i–ATP exchange reaction, believed to represent a steady-state reaction between ATP and P_i and utilizing the same enzymic transphosphorylative sequence as the DNP-activated ATPase and the phosphorylation in diaphorase region coupled to electron transport, was also measured following various periods of riboflavin deprivation (Table V). No consistent trend was observed either in unsupplemented or FAD-supplemented mitochondrial preparations.

Galactoflavin, a flavin inhibitor, has been reported (12) to result in riboflavin deficiency in periods as short as 10 days when incorporated into the diet of rats. We have attempted to produce severe riboflavin deficiency by adding galactoflavin to riboflavin-free diets and feeding them to adult rats for periods of between 10 and 28 days. Such treatment resulted in liver mitochondrial preparations with generally lower flavin levels than those of animals placed on riboflavin free diets at weaning and, in addition, appeared to be able to do so in less time (Table VI).

Table VII contains the results of experiments in which oxidative phosphorylation with succinate, glutamate, and β-hydroxybutyrate as substrates was assayed employing liver mitochondrial preparations from rats fed galactoflavin for 10 to 28 days. P/O ratios of all the preparations approached theoretical limits with each substrate and no differences in phosphorylative efficiency approaching statistical significance were seen. However, significant differences were observed in electron transport rates from both glutamate and beta-hydroxybutyrate, especially after 15 and 28 days on galactoflavin-enriched, riboflavin-free diets, the control preparations being greater in each case. Respiratory control, reflected in the percentage stimulation of mitochondrial oxidations by the presence of an ADP generating system, was also less in the 15- and 28-day experimental animals.

The ATPases of the same mitochondrial preparations as those used in the experiments reported in Table VII were studied (Table VIII). Low spontaneous ATPase activity was seen in resting mitochondria (no flask additions)

TABLE V

Effect of dietary riboflavin deprivation on mitochondrial P_i–ATP exchange
(Each flask contained 200 mg eq. mitochondria, 30 μmoles ATP, 20 μmoles P^{32}-potassium orthophosphate (pH 7.4), 8 μmoles $MgCl_2$, and 100 μmoles Tris buffer (pH 7.4) in a final volume of 2 ml. Shaken in open 25-ml Erlenmeyer flasks at 20° C for 20 minutes. All values represent means from six experimental observations. Standard error of the mean in parentheses)

Exptl. series[a]	Weeks deprived	Flask additions	P_i exchanged, μmoles/mg N/hr	
			Control	Experimental
1	1	None	18.11(1.5)	14.72(1.2)
		"	15.17(1.2)	14.06(0.9)
		FAD	15.11(1.0)	15.88(1.4)
		"	16.47(1.6)	16.87(1.1)
2	2	None	15.16(1.9)	20.63(1.4)
		"	11.77(2.1)	19.69(1.8)
		FAD	14.37(1.7)	20.48(1.4)
		"	13.55(1.1)	17.39(1.7)
3	3	None	16.68(1.6)	20.76(1.9)
		"	13.58(1.7)	17.75(1.9)
		FAD	17.03(1.4)	21.31(2.1)
		"	11.24(2.7)	19.79(1.7)
4	4	None	15.37(1.2)	17.00(1.6)
		"	17.49(1.6)	15.78(1.9)
		FAD	15.66(1.4)	18.36(1.7)
		"	16.67(1.1)	17.73(1.7)
5	5	None	8.64(0.7)	11.64(1.4)
		"	10.83(2.0)	15.66(1.4)
		FAD	11.46(1.3)	15.15(1.7)
		"	14.77(1.7)	16.92(1.5)
6	6	None	14.49(1.5)	17.36(1.1)
		"	13.32(1.9)	18.46(1.6)
		FAD	17.91(1.1)	18.46(1.3)
		"	14.79(1.5)	21.72(2.3)
7	12	None	12.31(0.9)	12.01(1.6)
		"	11.67(1.4)	13.21(1.7)
		FAD	10.86(1.6)	12.44(1.9)
		"	11.31(1.4)	11.69(1.2)
8	14	None	10.34(1.3)	12.31(0.9)
		"	12.44(1.8)	11.85(1.3)
		FAD	11.93(1.4)	12.56(1.3)
		"	12.12(1.7)	12.44(1.1)

[a]Series numbers correspond to those in Tables II, III, and IV.

from both galactoflavin-fed and control animals in the three dietary periods studied. The addition of DNP resulted in a somewhat greater stimulation of the ATPase activity of the experimental than of the control preparation, although the small difference was significant. The addition of magnesium alone stimulated the ATPase activity slightly, indicating that these preparations were not in a damaged state. The presence of magnesium and DOC stimulated ATPase activity and in this case, significant differences were observed between preparations from control rats and rats fed galactoflavin for 15 and 28 days. Although differences again were not of great magnitude, they were constant and indicated a deficient alternative ATPase activity in the 15- and 28-day galactoflavin-fed animals.

TABLE VI

Effect of galactoflavin feeding on mitochondrial flavin content
(All values represent means of six experimental observations.
Standard error of the mean in parentheses)

Days on galactoflavin diet	Flavin/1000 mg eq., mμmoles		Flavin/mg N, mμmoles	
	Control	Experimental	Control	Experimental
10	26.84*(2.1)	8.48*(0.92)	5.20*(1.4)	1.67*(0.37)
	23.38*(1.7)	8.88*(1.0)	6.46*(0.6)	2.34*(0.42)
15	34.46*(1.9)	6.52*(0.77)	6.89*(0.7)	1.18*(0.51)
	25.16*(2.2)	8.72*(0.91)	6.48*(0.7)	1.66*(0.38)
28	34.46*(1.4)	7.66*(0.67)	6.29*(1.1)	1.43*(0.92)
	31.56*(1.8)	7.24*(0.79)	6.11*(0.80)	1.37*(0.44)

*Indicates a statistically significant difference between control and experimental values. $P = 0.01$ or less.

Löw (9) has reported a biphasic response on atebrin of mitochondrial DNP-activated ATPase and an inhibition of magnesium-activated mitochondrial ATPase with increasing in vitro atebrin concentrations. We have repeated this type of experiment on both DNP-activated and magnesium-activated ATPases in order to confirm the experiments of Löw (9) and have also titrated DNP-activated ATPase with atebrin in mitochondrial preparations from galactoflavin fed rats and controls. Figure 1 contains results of a

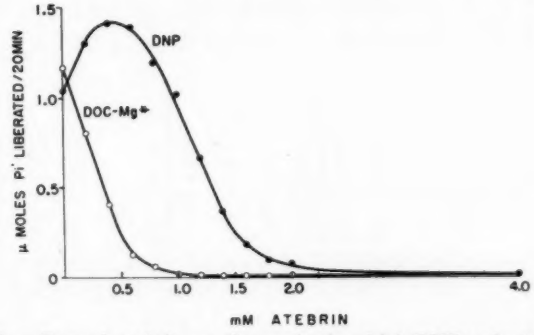

FIG. 1. The effect of atebrin on liver mitochondrial DNP-activated and Mg^{++}-activated ATPases. Flask contents and conditions of incubation as in Table IV.

typical experiment on atebrin titration of both types of ATPases in liver mitochondria from normal, untreated adult rats. Atebrin stimulates the DNP-activated ATPase at low concentrations and inhibits at high concentrations while the magnesium-activated ATPase is inhibited at all the atebrin concentrations tested. This is in direct confirmation of the results of Löw (9). Figure 2 contains the result of such an atebrin titration of DNP-activated ATPase of mitochondria prepared from the livers of control rats and rats fed galactoflavin for 10 days. Incorporation of galactoflavin into the diet for 15 or

TABLE VII

The effect of galactoflavin feeding on oxidative phosphorylation
(Conditions as in Tables I and III. β-OH, 60 μmoles/flask. All values represent means of six experimental observations. Standard error of the mean in parentheses)

Days on diet	Substrate	Flask additions	O_2 consumed, μatoms/mg N/hr		P/O	
			Control	Experimental	Control	Experimental
10	Succinate	None	40.47(3.1)	44.91(2.9)	1.96(0.07)	1.94(0.10)
	Succinate	Cyt. *c*, FAD	39.06(3.6)	46.74(3.1)	1.86(0.10)	1.92(0.06)
	Glutamate	None	68.67*(1.4)	56.22*(1.2)	2.42(0.07)	2.59(0.09)
	Glutamate	DPN, cyt. *c*, FAD	79.05*(1.9)	68.01*(1.5)	2.39(0.16)	2.51(0.11)
	β-OH	None	23.73(1.6)	24.51(1.8)	2.12(0.08)	2.22(0.04)
	β-OH	DPN, cyt. *c*, FAD	29.22(2.2)	25.98(1.6)	2.10(0.04)	2.19(0.06)
	Glutamate[a]	None, minus hexokinase (acceptor effect)	5.88(0.54)	7.10(0.76)		
15	Succinate	None	33.78(2.4)	26.85(1.8)	1.94(0.16)	1.93(0.09)
	Succinate	Cyt. *c*, FAD	36.93(1.2)	33.63(1.4)	1.90(0.10)	1.77(0.09)
	Glutamate	None	55.17*(0.9)	28.89*(1.1)	2.61(0.06)	2.61(0.08)
	Glutamate	DPN, cyt. *c*, FAD	57.57*(1.3)	38.37*(1.6)	2.58(0.14)	2.33(0.11)
	β-OH	None	19.47*(0.9)	12.86*(1.3)	2.49(0.05)	2.42(0.07)
	β-OH	DPN, cyt. *c*, FAD	22.20*(1.6)	13.05*(1.4)	2.37(0.07)	2.35(0.06)
	Glutamate[a]	None, minus hexokinase (acceptor effect)	5.80(0.47)	3.16(0.79)		
28	Succinate	None	29.74(1.9)	32.46(2.3)	1.82(0.09)	1.77(0.04)
	Succinate	Cyt. *c*, FAD	36.78(1.3)	34.47(1.8)	1.64(0.07)	1.71(0.05)
	Glutamate	None	40.95*(0.6)	35.73*(0.7)	2.42(0.08)	2.30(0.08)
	Glutamate	DPN, cyt. *c*, FAD	47.25*(0.9)	41.49*(1.0)	2.12(0.10)	2.08(0.05)
	β-OH	None	17.73*(0.8)	11.46*(0.8)	2.62(0.05)	2.66(0.05)
	β-OH	DPN, cyt. *c*, FAD	23.67*(1.2)	16.89*(0.8)	2.51(0.07)	2.44(0.04)
	Glutamate[a]	None, minus hexokinase (acceptor effect)	6.04*(0.26)	1.67*(0.14)		

[a]Values for the acceptor effect refer to the μatoms of O_2 consumed by a preparation in the presence of the hexokinase–glucose acceptor divided by the O_2 consumed in the absence of acceptor.
*Indicates a statistically significant difference between control and experimental values. $P = 0.01$ or less.

TABLE VIII

Effect of galactoflavin feeding on mitochondrial ATPases
(Conditions as in Table IV. All values represent means of six experimental observations. Standard error of the mean in parentheses)

Days on diet	Flask additions	P_i liberated, μmoles/mg N/hr	
		Control	Experimental
10	None	6.24(0.9)	7.51(0.7)
	DNP	29.82(1.2)	31.59(1.6)
	Mg^{++}, DOC	43.08(2.3)	37.89(2.1)
	Mg^{++}	11.01(1.8)	10.77(1.8)
15	None	5.88(0.7)	5.49(0.9)
	DNP	33.72*(1.1)	42.63*(0.9)
	Mg^{++}, DOC	63.72*(1.5)	48.33*(1.6)
	Mg^{++}	10.20(0.7)	8.70(1.0)
28	None	8.10(0.7)	6.78(0.8)
	DNP	41.60*(1.1)	49.17*(1.8)
	Mg^{++}, DOC	44.10*(1.6)	34.50*(1.6)
	Mg^{++}	8.10(1.1)	12.18(1.4)

*Indicates a statistically significant difference between control and experimental values. $P = 0.01$ or less.

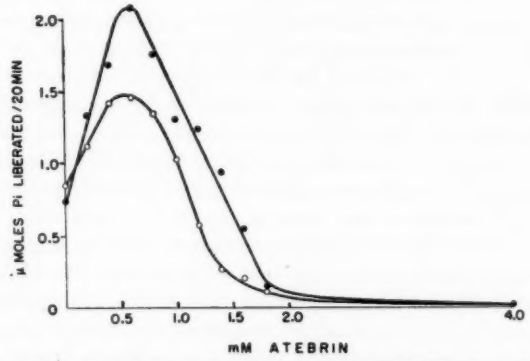

FIG. 2. The effect of atebrin on liver mitochondrial DNP-activated ATPase from control (○) and galactoflavin (●) fed rats. Flask contents and conditions of incubation as in Table IV.

28 days results in differences of similar magnitude. It may be seen that low concentrations of the flavin antagonist stimulate the DNP-activated ATPase of galactoflavin-fed rats to higher enzymic levels than it does the control preparation. Higher concentrations of atebrin are required to lower activity to near minimal activity in the galactoflavin-fed preparations than in the control.

The P_i–ATP exchange reaction was measured in mitochondrial preparations from livers of control rats and rats fed galactoflavin for 10, 15, and 28 days (Table IX). Although the control preparations appear to possess slightly greater exchange activity, these small differences do not approach statistical significance and may not be considered as indicating a trend.

TABLE IX

The effect of galactoflavin feeding on P_i–ATP exchange rate
(Conditions as in Table V. All values represent means of six experimental observations. Standard error of the mean in parentheses)

Days on diet	Flask additions	P_i exchanged, μmoles/mg N/hr	
		Control	Experimental
10	None	20.46(1.1)	20.34(1.3)
	"	19.89(1.4)	18.24(0.9)
	FAD	19.50(1.1)	19.72(1.2)
	"	18.90(1.4)	20.04(0.9)
15	None	23.34(1.2)	20.10(1.0)
	"	26.79(1.5)	20.16(1.1)
	FAD	23.70(1.3)	19.83(0.9)
	"	21.78(1.3)	20.01(1.3)
28	None	22.59(1.4)	20.70(0.9)
	"	22.14(1.4)	19.86(1.1)
	FAD	21.78(1.1)	19.59(1.3)
	"	20.97(1.2)	20.01(1.2)

Discussion

One of the central physiological problems confronting the biologist today concerns the nature of the three sites of energy conservation in the mitochondrial electron transport chain and the mechanism by which such energy transformation occurs. At present, three possible electron transport components are being seriously considered as candidates for such a coupling site. They are DPN (17), flavin (2), and certain substituted quinones (18). We have attempted to obtain information concerning flavin participation by studying oxidative phosphorylation and related reactions employing mitochondria isolated from livers of flavin-deficient animals as well as animals fed a flavin antagonist, galactoflavin. Prior to performing such experiments, it was thought desirable to ascertain the effect of fasting on mitochondrial flavin content and oxidative phosphorylation since it is known that animals on various deficient diets consume less food than normal. Using glutamate as substrate no difference in the P/O ratios between normal and 4- to 7-day starved rat liver mitochondria was observed, nor was the reducible flavin content of such particulate preparations different when calculated on a milligram nitrogen basis. The fact that the flavin content of liver mitochondrial preparations from starved animals appeared higher when referred to mitochondria derived from a given weight of fresh tissue might be explained by assuming a loss of water and glycogen from liver in starved animals and consequently higher apparent flavin values. Thus it was concluded that pair-fed controls were not necessary in flavin deficiency experiments involving oxidative phosphorylation since severe inanition did not appear to influence this metabolic system in our assay procedure. The absence of an observable defect in oxidative phosphorylation in mitochondria from rats fasted for 7 days was surprising in view of the reports that mitochondria *in situ*, following histological

fixation and staining, appear to go through a transition from filamentous to rod to sphere shape during starvation (19, 20, 21). It should be noted, however, that mitochondria may display an amazing degree of stability under rather harsh experimental treatment (22).

Since the experiments reported herein were designed to provide information concerning flavin involvement in energy conservation in mitochondria using a dietary deficiency approach, it was necessary that riboflavin deficiency should result in a significant lowering of the mitochondrial flavin level. It appears that a portion of the mitochondrial flavin is a very labile constituent of these particles while another portion is rather stable. For example, after 1 week of dietary riboflavin deprivation, the flavin content of the experimental mitochondrial preparations was approximately 58% of the control while after 14 weeks on the experimental diet the flavin content had fallen only an additional 25% to a value of 33% of the control level. The loss of flavin from such particulate preparations tends to confirm the experiments of Burch *et al.* (23), who have reported a lowering of FAD and FMN levels of liver tissue following 12 to 13 weeks of riboflavin deprivation. Since these authors report that in liver, under such conditions, FMN falls to approximately 8% of its original value while FAD falls to about 44% of its original value, it is interesting to speculate that the more labile component of the mitochondrial flavin might be FMN while the more stable might be FAD.

Although the customary approach to the assay of the yield of oxidative phosphorylation has been to measure the P/O ratio of isolated mitochondria in vitro under rigidly defined conditions, the concept has emerged recently that the DNP-activated ATPase as well as the P_i–ATP exchange reaction catalyzed by these particles represents a reaction sequence identical with that of the phosphorylation coupled to electron transport in the diaphorase region between DPN and cytochrome *b* (4). Consequently, in addition to measuring the efficiency of oxidative phosphorylation (P/O) of liver mitochondrial preparations from flavin-deficient rats, we have measured the DNP- and Mg^{++}-activated ATPases and the P_i–ATP exchange reaction. P/O ratios with either succinate or glutamate as substrate were normal for preparations obtained from animals subjected to 1 to 14 weeks of dietary riboflavin deprivation and no inhibition of glutamate or succinate oxidation was observed corresponding to the lowered mitochondrial flavin content. This is not entirely in accord with the results of Burch and Hunter (24), who have reported recently, in abstract form, that liver mitochondria from riboflavin-deficient rats display a lowered phosphorylation efficiency and an impaired oxidation of beta-hydroxybutyrate and succinate while DPNH dehydrogenase was normal and TPNH dehydrogenase fell to one-half the normal level. These authors suggest "that possibly flavoproteins other than the DPNH dehydrogenase may be involved in coupling of phosphorylation to the oxidative process". However, since the oxidation of glutamate is DPN-dependent and gives rise to three phosphorylations it must be assumed that the first

energy conservation site is involved in the over-all process. Since both the data in the present paper and those reported by Kielly (25) indicate a normal rate of glutamate oxidation and oxidative phosphorylation in riboflavin-deficient rat liver mitochondria, it would appear that the reduced flavin content of such mitochondria does not result in a lowered rate of electron transport in the diaphorase flavin region. However, upon more severe flavin depletion, such as that resulting from galactoflavin feeding, such defects in DPN-dependent oxidations may be seen (Tables VI and VII). It should not be assumed that the degree of flavin deficiency produced in these animals on riboflavin-deficient diet does not result in some mitochondrial alteration, however, since Pratt (26) has described definite morphological alterations resulting from such treatment.

Such an interpretation, however, does not preclude the possible participation of flavin in the transphosphorylation process between the coupling site and the final synthesis of ATP. Although the lack of differences in data derived from P_i–ATP exchange rates in mitochondria from both riboflavin-deficient and galactoflavin-fed rats on the one hand and normal rats on the other do not support such a possibility, some indication of differences was seen in experiments on the ATPase activities of these two preparations. Beginning with the sixth week of riboflavin deprivation and continuing through the 12th and 14th weeks, the DNP-activated ATPase activity of the experimental preparation is significantly greater than the control. The same relationship is seen in the DNP-activated ATPase of the preparation obtained from rats fed galactoflavin for 15 and 28 days. The Mg^{++}-activated ATPase activity of the galactoflavin-fed preparations indicates the opposite tendency in the control preparation, which shows a significantly greater activity than does the experimental. These seemingly divergent results may possibly be explained in terms of the biphasic response of ATPase to flavin inhibitors reported by Löw (9) and seen in Fig. 1. It is suggested that the lowering of the mitochondrial flavin content to the extent seen in the preparations from rats on riboflavin deficient diets results in a stimulation of the DNP-activated ATPase which would correspond to the stimulatory phase of the DNP curve in Fig. 1. Upon a greater depression of mitochondrial flavin, the magnesium-activated ATPase is affected and thus the inhibition seen in Fig. 1 would roughly correspond to such a situation. At the same time, the DNP-activated ATPase in the more severely flavin depressed particle might still be in the stimulatory phase of the curve. The results of experiments depicted in Fig. 2 would tend to support such an explanation since the DNP-activated ATPase of preparations from galactoflavin-fed rats appears to be more sensitive to the stimulatory effect of atebrin.

It is interesting to speculate on the possible reason for the biphasic DNP-activated ATPase response to increasing concentrations of atebrin. Löw (9) has suggested that the inhibitor, atebrin, causes a stimulation at low concentrations by shifting of a rate-limiting step in the DNP-activated

ATPase path and inhibits the reaction sequence at higher concentrations. Another possibility may be that at low concentrations of atebrin, which are not inhibitory, the association between substrate and enzyme may be accomplished with greater ease. An increase in mitochondrial membrane permeability would accomplish such an end. Such an interpretation may be indicated since the addition of atebrin to mitochondria, in the absence of DNP, activates resting ATPase to between 300 and 500% of the normal resting activity (9).

The lack of correlation between the lowering of mitochondrial flavin levels and the consistently normal P/O ratios in our experiments reported herein do not lend support to the idea brought forth by Lindberg *et al.* (2). However, it is entirely possible that the major portion of flavin lost during riboflavin deprivation and galactoflavin feeding is FMN and that the predominant residual flavin is FAD, possibly the diaphorase moiety responsible for the process of energy conservation. If such is the case, and if it is subsequently found that flavin does participate in the transformation of energy from electron transport to the synthesis of ATP, then it must be assumed that such a centrally vital component to the maintenance of homeostasis in energy metabolism enjoys a preference in stability during the process of flavin loss accompanying riboflavin deficiency.

Acknowledgment

The authors wish to thank Mrs. Joanne Maia for her skilled technical assistance.

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THE EFFECTS OF AN ANTIHISTAMINE (PROMETHAZINE) ON THE REACTION OF TENDONS TO TRAUMA— A HISTOLOGICAL STUDY[1]

F. G. WALKER, SYLVIA H. BENSLEY, AND W. K. LINDSAY

Abstract

As a part of a continuing study on the healing of flexor tendons, the effects of an antihistamine–antiserotonin compound (promethazine) were studied in 109 chickens. A form of standard surgical trauma to the intact flexor profundus tendon was devised, and the results of several different methods of administration of promethazine were compared. These studies indicate that minimal surgical trauma applied to an intact flexor tendon produces a marked reaction consisting of a degenerative phase, a phase of dedifferentiation, and a phase of regeneration and maturation. The histological studies reveal that such highly specialized intercellular substances as collagenic fibers and bundles, though non-living, are not inert and permanent structures. They can break down, completely disappear, and be reformed as a result of the reaction to trauma of specialized tenocytes and undifferentiated mesenchymal cells of the tendon and epitenon. The effects of an antihistamine (promethazine) are to prolong the phase of degeneration and loss of differentiation and, upon withdrawal, to accelerate the phase of growth and maturation. These effects vary with the dose and method of administration of promethazine.

Introduction

The effects of antihistamine drugs on many physiological and pathological processes have been studied, but there have been few reports on the effects of such drugs on fibroplasia. Berman *et al.* (1) found that methapyrilene HCl (Histadyl (R)) limited the amount of fibroplasia in experimental soft tissue wounds produced by severe or continued trauma. This effect was not observed in incised wounds where there had been minimal damage to cells. Hormia and Hormia (2) found that wound healing in the rat was slowed slightly by the use of chlorpromazine (Largactil (R)); this effect was not statistically significant. Weil *et al.* (3) reported that pyrrobutamine (Pyronil (R)) retarded the healing of corneal incisions in the rabbit. Conversely, Baldridge (4) reported pyrrobutamine produced a significant increase in the rate of healing of wounds in the guinea pig. There have been no reports of the effects of such drugs on tendon healing.

As part of a continuing study on the healing of flexor tendons (5), the effects of an antihistamine on the healing of traumatized chicken tendons were studied. Promethazine HCl (Phenergan (R)) was chosen for this experiment because it possesses potent antihistamine activity (6). It also antagonizes the action of serotonin (7). Histological aspects of this study are presented.

[1]Manuscript received July 4, 1960.
Contribution from the Research Institute, The Hospital for Sick Children, Toronto; Department of Anatomy, University of Toronto; and the Department of Surgery, The Hospital for Sick Children, Toronto, Ontario.

Methods

One hundred and nine female chickens, unselected as to breed, 6 to 8 months of age, and weighing from 2300 to 3400 g were used throughout the experiment. They were fed a standard diet consisting of scratch grain, egg pellets, egg mash, grit, and oyster shell, and were given water ad libitum.

Preoperatively, chickens were starved for 18 hours. Sedation was obtained with pentobarbital sodium (Nembutal (R)) I.M. 20 mg/kg. Regional anaesthesia was obtained by using lidocaine 1% (Xylocaine (R)) as a femoral and sciatic nerve block. A tourniquet was applied to each foot prior to the surgical procedure.

A standard operation was carried out to obtain a surgical control. Under surgically sterile conditions, using an L-shaped incision, a palmar skin flap was reflected from the center toe, the flexor sheath was excised throughout the length of the toe, and the flexor sublimis tendon was excised from its insertion to as high in the foot as could be reached from the surgical exposure. Skin edges were then apposed with interrupted 0000 silk sutures.

Standard surgical trauma was produced using a similar surgical exposure. In addition, a 0000 silk suture on a No. 14 curved cutting needle with a French eye was inserted 18 times into the intact flexor profundus tendon (Fig. 1). The suture was then removed prior to apposing skin edges. The tendon was held as necessary with toothed Adson forceps (Ingram and Bell, Catalogue No. 533).

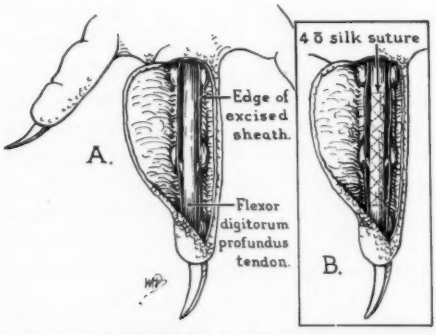

FIG. 1. (A) Control procedure, flexor sheath and flexor sublimis tendon excised. (B) Standard surgical trauma: 0000 silk inserted 18 times into intact flexor profundus tendon.

A combination of the insertion and removal of the silk suture plus the handling of the tendon with toothed Adson forceps produced a zone of standard tendon trauma 1 cm on each side of the attachment of the vinculum longum, free from possible foreign body reaction. The surgical control procedure duplicated the standard surgical trauma procedure in all respects except for the administration of trauma to the intact flexor profundus tendon.

Both feet in all chickens were subjected to a surgical procedure. In certain groups the tendon of the long toe on the other foot was divided transversely and repaired with 000000 stainless steel wire. The results from this experiment are described elsewhere (8).

Postoperatively, both feet were immobilized in plaster of paris casts for 3 weeks, or until the experiment was terminated. After removal of the casts the chickens were allowed to walk freely, and were perched for 4 to 6 hours per day on a 1 in. diameter rod. Skin sutures were removed 4½ weeks postoperatively.

The effects of several methods of drug administration were compared as follows.

Group I: Surgical control. No drug was administered.

Group II: Standard trauma. No drug was administered.

Group III: Standard trauma. Promethazine 15 mg (0.6 cc) was instilled around the tendon immediately after the skin closure, using a No. 27 hypodermic needle inserted between the skin edges.

Group IV: Standard trauma. Promethazine I.M., 100 mg, was administered into the pectoral muscles at 8 a.m., 12 noon, and 4 p.m. on the day of operation only. Operation was carried out in the early afternoon.

Group V: Standard trauma. Promethazine I.M. 25 mg b.i.d. was administered into the pectoral muscles for 7 days postoperatively.

Group VI: Standard trauma. Promethazine I.M. 25 mg b.i.d. was administered into the pectoral muscles for 7 days postoperatively. Promethazine 15 mg (0.6 cc) was instilled around the tendon immediately after skin closure, as in group III.

Group VII: Standard trauma. Promethazine I.M. 100 mg was administered three times on the day of operation in a manner similar to group IV. Promethazine I.M. 25 mg b.i.d. was given for 30 days postoperatively.

Group VIII: Standard trauma. Promethazine I.M. 100 mg was administered three times on the day of operation and promethazine I.M. 25 mg b.i.d. was given for 30 days postoperatively (i.e. the same regimen as group VII). Promethazine 15 mg (0.6 cc) was instilled around the tendon immediately after the skin closure, as in group III.

This enabled us to study the effects of standard surgical trauma alone (group II), and compare the effect on the reaction to standard surgical trauma of topical promethazine (group III), early administration of promethazine (group IV), two ranges of prolonged administration (groups V and VII), and combinations of these (groups VI, VII, and VIII).

In groups VII and VIII toxic levels of the drug were reached. Many of the chickens in these groups became lethargic, developed diarrhoea, lost weight, and developed pressure sores over the sternum and in the ankle creases.

Birds were killed by cervical dislocation at intervals of 48 hours, 1, 2, 3, 5, 8, and 16 weeks in all groups. At certain time intervals two or more birds were sacrificed. This was necessitated by illness of the bird and occurred mainly in groups VII and VIII. Most time intervals were represented by a single specimen. The feet were amputated and fixed in 10% formalin. Autopsy dissection of the toe subjected to operation was carried out and a segment of the tendon extending 1 cm on each side of the vinculum longum was removed. This segment was embedded in paraffin and sectioned transversely through the area of attachment of the vinculum longum. All sections were cut at five microns and stained routinely with hematoxylin and eosin. To facilitate comparison, photomicrographs (×64) were taken of all sections using Ectachrome EF 135 film.

Cell counts were carried out under oil immersion using a 10× ocular and a 90× objective. In each section studied, all nuclei, except those in vessels and vessel walls, in 10 fields within the tendon proper were counted.

The largest and smallest collagenic fibers were selected in the tendon in each section studied and the largest and smallest cross-sectional diameter of each was measured in microns using an ocular micrometer. The average of these two diameters of each fiber was used to represent the size of the fiber.

Observation

Terminology

The microscopic anatomy of the normal flexor tendon is shown schematically (Fig. 2). The *epitenon* is a cellular layer completely enveloping the tendon. The endotenon is continuous with it and projects through the tendon substance, dividing it into *tendon bundles*. Like the epitenon, the endotenon is moderately cellular. It contains an occasional blood vessel. Tendon bundles are composed of *collagenic fibers* and fibrocytes which may be termed *tenocytes*. Mature collagenic fibers have regular outlines, and the tenocytes have little visible cytoplasm.

Group I: (Surgical Control, No Drug)

The microscopic anatomy of sections in the surgical control group is essentially normal. The epitenon is two or three cells in thickness; the endotenon is conspicuous, moderately cellular, and contains some vascular channels. The tendon bundles are well outlined, and contain collagenic fibers which have a regular border. Tenocytes are seen uniformly throughout the tendon. They have little visible cytoplasm. A typical section, taken 2 weeks after surgery, is illustrated (Fig. 3).

Average cell counts at all intervals studied after surgery are relatively constant (Table I), as are average diameters of largest and smallest collagenic fibers (Table II).

Group II: (Standard Trauma, No Drug)

Forty-eight hours (Fig. 4).—The epitenon is thin. Immediately outside it is

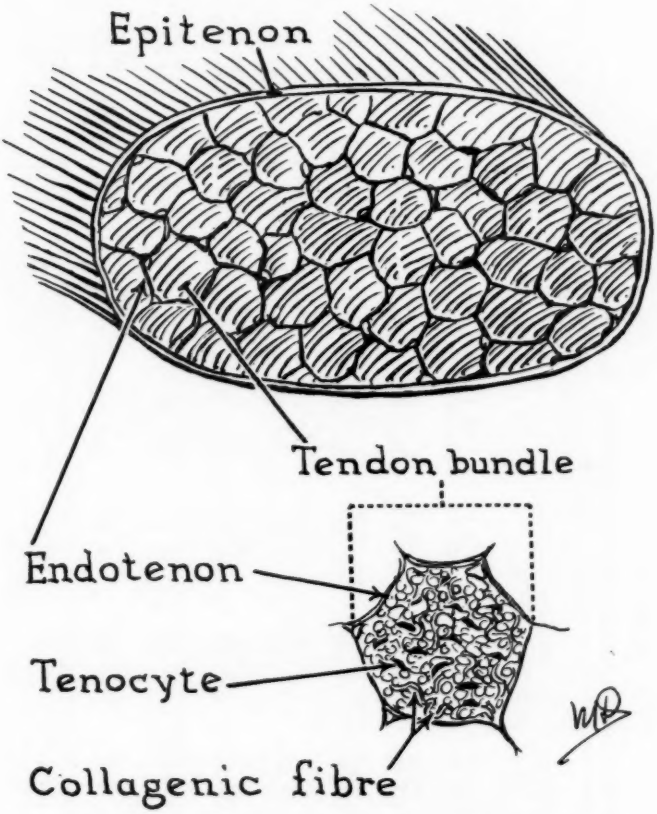

FIG. 2. Schematic cross section of normal flexor tendon.

seen an acute inflammatory exudate. The endotenon is conspicuous, cellular, and vascular. Tendon bundles are well outlined. Many collagenic fibers are larger than those in the surgical control group; some are very small; most are frayed, i.e. the fiber borders are irregular and frond-like. This is more obvious with low illumination. Total cellularity in the cross-sectional area of the tendon proper is not increased. In the area of the largest fibers, there is a patchy loss of nuclei.

One week (Fig. 5).—The epitenon is thick and cellular. The endotenon, which is poorly defined, shows increased vascularity and cellularity. Tendon bundles are partially replaced by cellular tissue. Collagenic fibers are frayed and small, although in some areas they are of normal diameter. The total number of nuclei is slightly increased.

Two weeks (Fig. 6).—The epitenon is relatively thin but is quite cellular and

TABLE I

Average cell counts (10 fields) of all nuclei except those in vessels and vessel walls. Range is given in parentheses

	Hours,	Weeks					
	48	1	2	3	5	8	16
Group I: (Surg. cont.)	30.2 (20–40)	30.9 (20–41)	34.0 (32–52)	28.1 (18–40)	48.1 (36–64)	32.8 (18–52)	64.1 (44–80)
Group II: (Standard trauma)	30.2 (18–46)	72.5 (40–130)	120.8 (85–215)	87.9 (16–164)	216.8 (144–256)	190.8 (140–240)	170.1 (140–204)
Group III: (Promethazine top. (15 mg))	30.5 (24–38)	27.4 (10–48)	29.3 (16–40)	24.1 (10–43)	151.3 (59–214)	113.8 (88–144)	52.0 (15–77)
Group IV: (Promethazine I.M. 1 day (300 mg))	28.2 (19–41)	31.8 (21–44)	82.4 (62–115)	29.7 (20–44)	85.6 (65–151)	327.0 (160–400)	147.2 (90–192)
Group V: (Promethazine I.M. 1 week (350 mg))	30.9 (18–42)	24.6 (18–42)	66.3 (52–99)	71.2 (44–120)	93.9 (51–120)	335.6 (318–352)	288.1 (230–402)
Group VI: (Promethazine top. (15 mg); promethazine I.M. 1 week (350 mg))	32.6 (24–48)	19.9 (10–28)	26.0 (8–43)	33.9 (16–60)	4.7 (0–23)	133.1 (89–188)	61.0 (33–82)
Group VII: (Promethazine I.M. 1 month (1800 mg))	30.5 (21–37)	31.2 (21–44)	19.4 (9–36)	18.5 (15–21)	222.8 (138–278)	130.0 (104–158)	42.2 (15–68)
Group VIII: (Promethazine top. (15 mg); promethazine I.M. 1 month (1800 mg))	33.7 (29–52)	30.2 (20–38)	37.6 (28–45)	5.3 (2–11)	294.4 (256–328)	274.8 (200–452)	223.5 (194–282)

TABLE II

Average collagenic fiber size in microns

	Hours, 48		Weeks											
			1		2		3		5		8		16	
	L*	S*	L	S	L	S	L	S	L	S	L	S	L	S
Group I: (Surg. cont.)	2.70	0.90	2.20	1.00	2.37	1.13	2.40	1.20	3.55	1.00	2.95	0.97	2.05	1.22
Group II: (Standard trauma)	4.55	0.47	1.65	0.27	2.87	0.40	2.65	0.40	1.50	0.37	0.10	5.34	1.55	0.65
Group III: (Promethazine top. (15 mg))	3.30	0.57	4.47	0.45	4.15	0.60	3.85	0.32	1.45	0.32	1.25	0.40	5.25	0.55
Group IV: (Promethazine I.M. 1 day (300 mg))	2.65	1.70	4.25	1.85	3.60	0.60	2.50	0.50	3.20	0.47	2.75	0.40	2.90	0.27
Group V: (Promethazine I.M. 1 week (350 mg))	2.07	0.45	2.37	0.40	2.85	0.35	2.67	0.22	2.00	0.25	0.80	0.37	2.65	0.30
Group VI: (Promethazine top. (15 mg); promethazine I.M. 1 week (350 mg))	3.25	0.60	2.70	0.45	3.85	0.35	4.40	0.40	3.67	0.47	2.00	0.40	2.20	0.35
Group VII: (Promethazine I.M. 1 month (1800 mg))	2.77	1.52	4.75	1.67	8.30	0.35	3.35	0.45	5.80	0.42	1.80	0.47	2.17	0.52
Group VIII: (Promethazine top. (15 mg); promethazine I.M. 1 month (1800 mg))	3.27	0.57	3.67	0.45	2.90	0.35	4.12	0.42	3.47	0.32	2.00	0.40	3.02	0.47

*L = largest; S = smallest.

very vascular, as is the endotenon. Many of the tendon bundles are replaced by cellular tissue. A very few areas show typical bundles. There are numerous nuclei in these areas. Almost all of the collagenic fibers are small; where typical bundles are seen they are of normal size. All fibers are frayed.

Three weeks (Fig. 7).—The epitenon is several cells in thickness, and is moderately cellular and vascular, as is the endotenon, which is conspicuous. Most tendon bundles are distinct but show a marked increase in the number of nuclei contained within them. Mitoses are seen in these areas. In some areas nuclei are not increased in number; collagenic fibers in these areas are of normal size. Where nuclei are increased, fibers are small. All fibers are frayed.

Five weeks (Fig. 8).—The epitenon is not seen as an entity. The endotenon where recognizable is thick and cellular. It demarcates very cellular areas which are not recognizable as tendon bundles. The entire tendon is very vascular. Almost all of the collagenic fibers are very small and frayed, while a very few normal-sized fibers are seen. These, too, are frayed.

Eight weeks (Fig. 9).—A thin layer of cells, suggestive of a primitive epitenon, surrounds the tendon. Tendon bundles and fibers are completely replaced by cellular tissue. Within this, near the center of the tendon, there is a small area of fine collagenic fibers which stain more deeply and have a smooth outline. Associated with these fibers are cells with hyperchromatic nuclei and visible cytoplasm which envelops a fiber.

Sixteen weeks (Fig. 10).—The epitenon and endotenon are cellular and vascular. The tendon bundles are small, as are most of the collagenic fibers. The fibers are fairly well oriented linearly, and have a smooth border. The total number of nuclei themselves is small.

Cellularity and Fiber Size: (Table I, Table II)

Average cellularity, as measured by cell counts taken in the cross-sectional area of the tendon proper, increases with time to a maximum at the fifth week. It then decreases slightly until the 16th week.

The average diameter of the largest collagenic fiber is increased 48 hours after standard trauma. It then falls steadily until the fifth week, to return to normal levels or slightly higher by 16 weeks. Very small fibers are seen as early as 48 hours after operation and fibers smaller than normal are seen at all times studied after standard surgical trauma.

Group III: (Standard Trauma: Promethazine 15 mg Topically)

Forty-eight hours, 1, 2, and 3 weeks after standard surgical trauma, the general architectural pattern of the tendons studied varies little from normal. Collagenic fibers are generally larger than normal and have frayed borders. Five and eight weeks after trauma, the histology is similar to that seen in group II at 2 weeks. Sixteen weeks after operation, the general architectural pattern is similar to that of group II at the same time interval. Cellularity is less, and fiber size is generally greater. Very few frayed fibers are seen at all intervals studied.

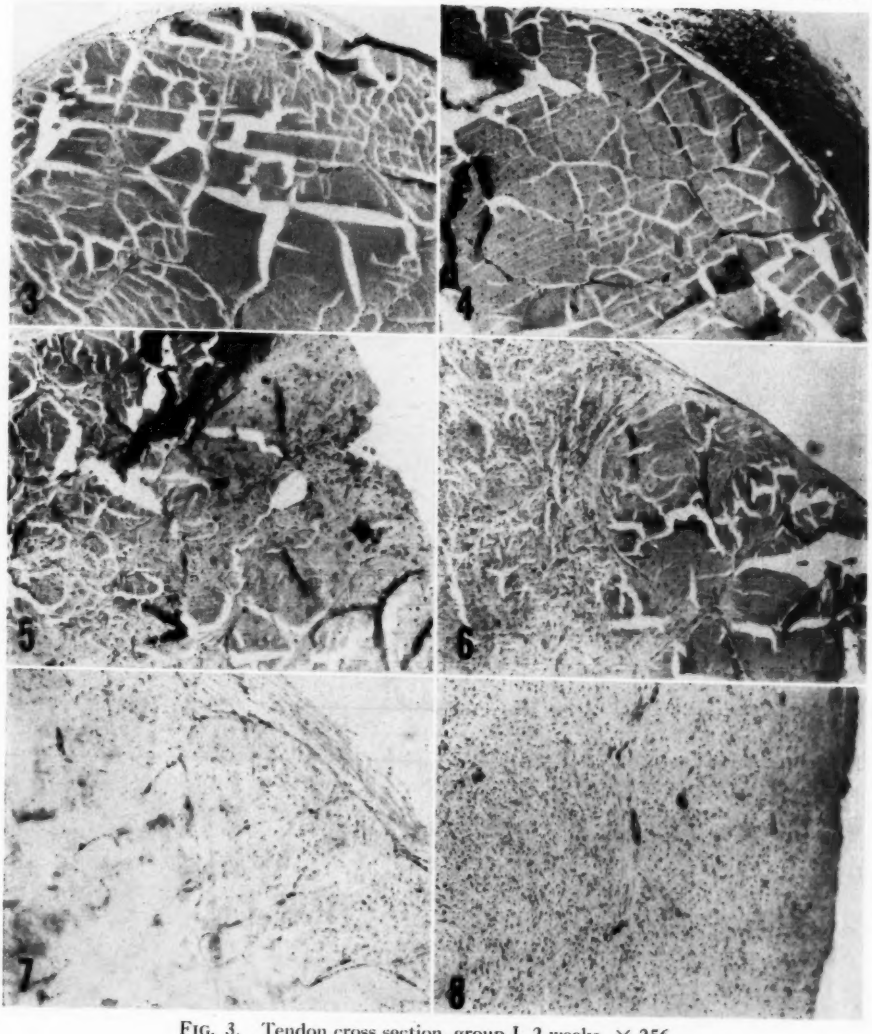

FIG. 3. Tendon cross section, group I, 2 weeks, × 256.
FIG. 4. Tendon cross section, group II, 48 hours, × 256.
FIG. 5. Tendon cross section, group II, 1 week, × 256.
FIG. 6. Tendon cross section, group II, 2 weeks, × 256.
FIG. 7. Tendon cross section, group II, 3 weeks, × 256.
FIG. 8. Tendon cross section, group II, 5 weeks, × 256.

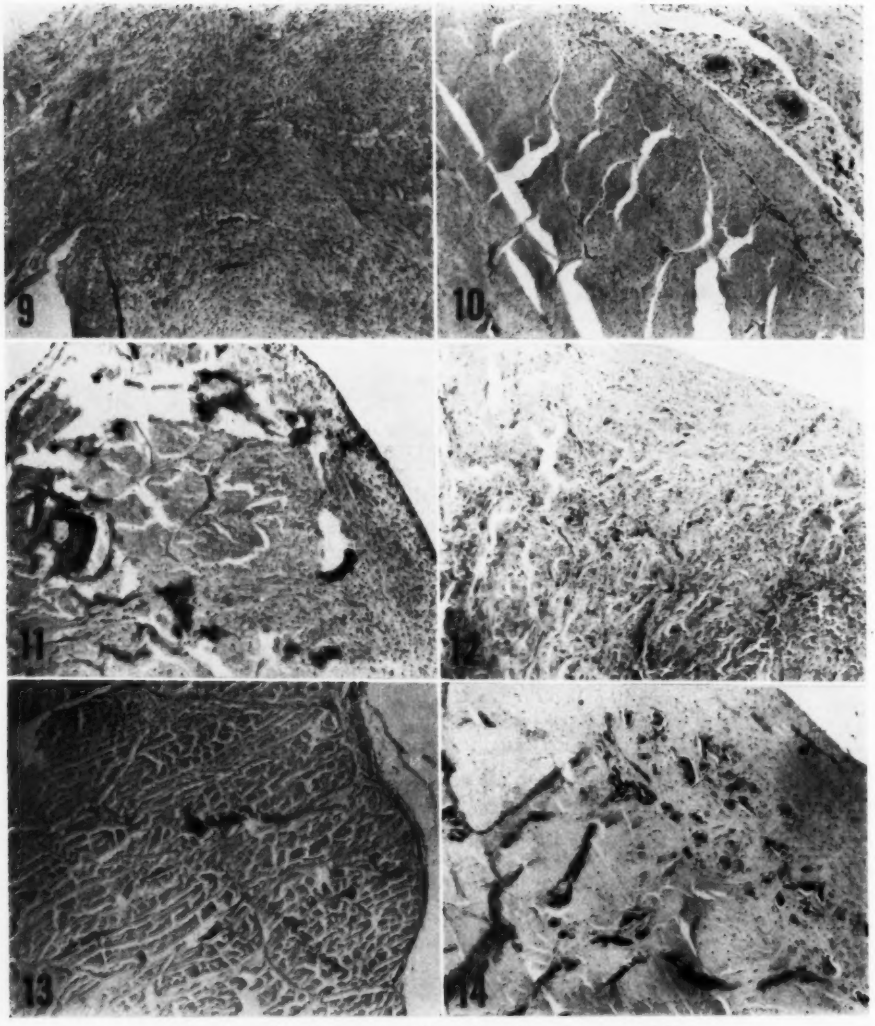

FIG. 9. Tendon cross section, group II, 8 weeks, × 256.
FIG. 10. Tendon cross section, group II, 16 weeks, × 256.
FIG. 11. Tendon cross section, group IV, 5 weeks, × 256.
FIG. 12. Tendon cross section, group IV, 8 weeks, × 256.
FIG. 13. Tendon cross section, group V, 3 weeks, × 256.
FIG. 14. Tendon cross section, group VII, 5 weeks, × 256.

Group IV: (Standard Trauma; Promethazine I.M. 300 mg Given on Day of Operation)

Forty-eight hours, 1 and 3 weeks after standard trauma, the architectural pattern of the tendon is normal, but collagenic fibers are generally large and frayed. No very small fibers are seen before 2 weeks. At 2 and 5 weeks after trauma, the histology is similar to that seen in group II at 1 week (Fig. 11). At 8 weeks, the microscopic anatomy is similar to that in group II, 8 weeks, but many more small well-stained fibers are seen enveloped by cytoplasm and accompanied by hyperchromatic nuclei (Fig. 12). The picture seen at 16 weeks is similar to that of group II, 16 weeks.

Group V: (Standard Trauma; Promethazine I.M. 350 mg over 7-Day Period)

The general architectural pattern is near normal for the first 5 weeks after standard trauma (Fig. 13). Fiber size and total cellularity in the cross-sectional area of the tendon proper is normal for the first 3 weeks; most fibers are frayed; very small fibers are seen early. Fibers are small and markedly frayed at 5 weeks, and cellularity is increased. Eight weeks after operation, the tendon resembles that in group II, 5 weeks. The architecture is completely obliterated by very cellular, vascular tissue. The picture at the 16-week interval is similar to that of group II, 16 weeks, although cellularity is greater.

Group VI: (Standard Trauma; Promethazine I.M. 350 mg over 7-Day Period; Promethazine 15 mg Topically)

Forty-eight hours to 3 weeks after trauma, tendons show a normal architectural pattern. Cellularity is normal; fibers are generally large and frayed. Five weeks after trauma, there is a marked decrease in the total number of nuclei seen within the tendon proper, although the architectural pattern is normal. The sections at 8 weeks are similar to those of group II at 2 weeks. The epitenon and endotenon are nearly normal, but many tendon bundles are replaced by cellular, vascular tissue. Sixteen weeks after trauma the microscopic anatomy seen in group II at 16 weeks is repeated, although total cellularity is less. At all times studied small fibers are seen.

Group VII: (Standard Trauma; Promethazine I.M. 1800 mg over 30-Day Period)

The architectural pattern and cellularity are normal for the first 3 weeks. Very large, frayed fibers are seen during this period, while small fibers are not seen until the second week. A peculiar picture is seen 5 weeks after standard trauma. The epitenon is thickened and cellular. The endotenon is not conspicuous. Tendon bundles are small and contain an increased number of poorly stained nuclei. Most fibers are small and frayed, while some are very large. Many relatively large vessels are seen scattered through the tendon without relationship to the endotenon. The entire tendon stains poorly (Fig. 14). Sections at 8 weeks are similar to those of group II, at 8 weeks. As in group IV, 8 weeks, many small well-stained fibers are seen, associated with large well-stained nuclei and enveloped by cytoplasm. Tendon architecture,

fibers, and total number of nuclei seen 16 weeks after trauma are all nearly normal.

Group VIII: (Standard Trauma; Promethazine I.M. 1800 mg over 30-Day Period; Promethazine 15 mg Topically)

For the first 2 weeks after standard trauma tendon architecture is normal, as is the cellularity. Collagenic fibers are generally large and frayed. Small fibers are seen at 1 week. At 3 weeks, the epitenon and endotenon are conspicuous, and tendon bundles are distinct. There is a paucity of nuclei throughout the entire tendon and the collagenic fibers, which are generally large and frayed, stain poorly. Five weeks after operation, the appearance is similar to that in group II at 2 weeks, and at 8 weeks the tendon resembles that in group IV and group VII, at 8 weeks. The specimens at 16 weeks are similar in all respects to those in group II at 16 weeks.

Discussion

Surgical trauma was recognized to be detrimental to tendon healing as early as 1918 (9), and this fact has been reiterated by many authors. From this study it is evident that minimal surgical trauma to the intact tendon (designated here as "standard trauma") is sufficient to produce an inflammatory reaction and complete loss of normal tendon structure. This finding has made possible the study of the mechanisms and period of time involved in the degeneration and regeneration during healing of such a highly specialized connective tissue as tendon.

As early as 48 hours after standard trauma, there is swelling of collagenic fibers rapidly accompanied by a fraying or dissolving of their margins. Microscopically the fibers have a waxy appearance. The swelling probably represents a rapid uptake of water due to altered pH or electrolyte content of the fibers brought about by enzymes, H-substances, or metabolites released from the injured tissue (10). The frayed appearance of the fiber border probably represents a more rapid loss of interfibrillar matrix than of the fibrila. This may be due to several factors: the hydration and depolymerization of the matrix, the action of hyaluronidase or other metabolites released locally from the injured tenocytes, and, later, from the proliferating cells and the increased blood and tissue fluid in the more cellular and vascular endotenon.

Most fibers originally making up the tendon eventually disappear. This process lasts 5 to 8 weeks.

Cellular changes accompany changes in the fibers. Initially there is a patchy loss of nuclei, followed by an increase in over-all cellularity of the tendon which progresses to a maximum about the fifth week. The cellular proliferation comes from many sources. Cells in the epitenon and endotenon undoubtedly contribute, but many of the new cells arise from cells within the tendon bundles themselves, either from mature tenocytes or from resting primitive mesenchymal cells. One to three weeks after trauma we see increased cellularity in the tendon bundles themselves while the epitenon and endotenon are still

relatively acellular. Occasional mitoses are seen within the bundles at this time.

Cells begin to form new collagenic fibers sometime before the eighth week. Such cells have large plump nuclei and bluish cytoplasm. Intimately associated with the nuclei and partially surrounded by the cytoplasm are newly formed fibers. Initially the fibers are very small, have a smooth border, and stain deeply with eosin.

As the traumatized tendon regenerates, the fibers approach normal size and orientation, while the nuclei of the fibroblasts become smaller. This represents maturation. The epitenon and endotenon return to a resting state, even while cellularity within the bundles is increased. We may speculate that eventually many of these cells will disappear.

As would be expected, vascularity closely parallels the cellular response. Vascularity is first increased in the endotenon as early as 48 hours, and in the epitenon at 1 week. By 5 weeks, the entire tendon is very vascular. Vascularity then declines, but is still increased in the epitenon and endotenon at 16 weeks. Again we may speculate that with time it will return to normal levels.

Thus three phases of reaction to minimal surgical trauma can be recognized, although these phases overlap each other. They may be termed:

1. *The degenerative phase*, characterized by cell injury and death, with loss of nuclei, and swelling and fraying of collagenic fibers. It may be assumed that during this phase the mucopolysaccharides which serve to cement the collagenic bundles are being depolymerized.

2. *The phase of dedifferentiation*, characterized by breakdown of the collagenic fibers into the smallest visible fibers, and presumably a depolymerization of the collagen itself into polypeptides and amino acids, thus producing a shift from the formed to the amorphous type of intercellular substance. This is accompanied or followed by a loss of differentiation of surviving tenocytes and a proliferation of young fibroblasts, with increasing vascularity.

3. *The phase of growth and maturation*, characterized by the differentiation of fibroblasts, formation of new fibers, orientation and increase in size of new fibers, and finally a return to the normal size and structure of the tendon.

Promethazine *prolongs* the degenerative phase of the response to standard trauma and *delays* the phase of dedifferentiation. *Acceleration* of the phase of growth and maturation occurs upon withdrawal of the drug. This effect varies with the route and dosage of the drug administered. Topical promethazine, 300 mg given over 1 day, or 350 mg given over 1 week, have similar effects. Toxic dosages of promethazine (1800 mg given over 1 month), as well as prolonging the degenerative phase, delaying the phase of loss of differentiation, and, upon withdrawal, accelerating the phase of growth and maturation, alter the time relation of these to one another. This gives rise to bizarre histological findings. A similar bizarre sequence is seen when topical promethazine is added to the regimen.

The following may be a possible explanation of what occurs. In the tendon we find two types of connective tissue: the cellular, less differentiated epitenon

and endotenon, and the more differentiated collagenic fibers. The response of the less differentiated connective tissue to injury, whether it be physical or chemical, is the breakdown of cells and intercellular substances, with the release of histamine, serotonin, and other metabolites. These lead to an inflammatory reaction which consists of both proliferative and vascular phases, that is, the formation of granulation tissue. As an end result we have fibrosis, and possibly overmaturation. Highly specialized connective tissue such as is found in the collagenic fibers follows a similar pattern, except that instead of proliferation there is degeneration. Promethazine as an antihistamine compound may alter the intensity of the inflammatory response either by blocking the breakdown of cells and intercellular substances, by directly inactivating released H-substances, or by competing with them at receptor cells. Thus degeneration of collagenic fibers will be prolonged and there will be little or no stimulation for proliferation of cells. As the effects of the antihistamine wear off, degeneration, dedifferentiation, and maturation proceed at a rapid rate as promethazine itself, in certain concentrations, may act as an injuring agent, hence stimulate the reaction further.

Summary and Conclusion

These studies indicate that minimal surgical trauma applied to an intact flexor tendon produces a marked reaction consisting of a degenerative phase, a phase of dedifferentiation, and a phase of regeneration and maturation.

The histological studies reveal that such highly specialized intercellular substances as collagenic fibers and bundles, though non-living, are not inert and permanent structures. They can break down, completely disappear, and be reformed as a result of the reaction to trauma of specialized tenocytes and undifferentiated mesenchymal cells of the tendon and epitenon.

The effects of an antihistamine (promethazine) are to prolong the phase of degeneration and loss of differentiation and, on withdrawal, to accelerate the phase of growth and maturation. These effects vary with the dose and method of administration of promethazine.

Thus, promethazine has been a useful tool in analyzing the mechanisms of the response of tendon to a standardized trauma as well as indicating a possible approach to the problem of the repair of injured tendons and other highly specialized connective tissue structures.

Acknowledgments

Phenergan used in this experiment was kindly supplied by Poulenc Ltd., Montreal. This project was assisted by funds allocated by the Province of Ontario under the National Health Grants Programme of the Department of National Health and Welfare, Ottawa (Dr. Walker and Dr. Lindsay), and by the Banting Research Foundation (Dr. Bensley).

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THE SUCCINIC OXIDASE ACTIVITY OF RAT LIVER MITOCHONDRIA[1]

G. R. WILLIAMS

Abstract

Rat liver mitochondria diluted into a high-potassium phosphate-free medium containing 2×10^{-5} *M* DNP take up oxygen readily if succinate is added at the same time as the mitochondria. If the addition of succinate is delayed, the respiration elicited is markedly reduced, falling to about 5% of the zero-time control at 2 minutes. Succinate-linked respiration can be restored by the addition of ATP or of cysteine sulphinic acid. A spontaneous recovery occurs on prolonged incubation (>20 minutes). The inactivation, at least at such short incubation times, is dependent on the presence of DNP, and partial protection is provided by inorganic phosphate and the substitution of sucrose for KCl. Considerable protection is also provided by the presence of 1.5 m*M* Amytal. These results are all consistent with the hypothesis that oxaloacetate is produced from endogenous substrate during the first 1 or 2 minutes after dilution of the mitochondria, and that the failure to demonstrate succinic oxidase activity after this time is due to the inhibition of succinic dehydrogenase by oxaloacetate.

Introduction

The inhibition of succinic oxidase by oxaloacetate has been known for over twenty years (1, 2) and it has been recognized that the enzymic formation of oxaloacetate can thus complicate the study of succinic oxidase in certain systems (3, 4, 5). We have recently reported a failure of rat liver mitochondria to oxidize succinate in rather specific circumstances (6). It now appears that this failure is due to an accumulation of oxaloacetate produced from the substrates which accompany the mitochondria during their preparation. It is the purpose of this paper to discuss the circumstances under which such accumulation can occur.

Methods

Rat liver mitochondria were prepared in 0.25 *M* sucrose[2] in the conventional manner (7). The final suspension in sucrose was made at a volume of 1.0 ml for each 10 g of liver homogenized. Oxygen uptakes were measured in the polarographic device previously described (8, 9), the final volume in the rotating cell being 1.0 ml in all cases. In the present experiments the diffusion current was recorded from the output of a Keithley Model 603 electrometer amplifier with a 10^6-ohm resistance across the input. All experiments were carried out at pH 7.2 and at room temperature.

Results

The nature of our observations is clearly indicated in Fig. 1. It will be noted

[1]Manuscript received June 20, 1960.
Contribution from the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario. A preliminary account of these results has been presented (6).

[2]The mitochondria used in these experiments were isolated by Mrs. Myra Smithies and I should like to express my indebtedness to her for these preparations.

that succinate added at zero time brings about rapid uptake of oxygen as indicated by the downward slope of the polarographic trace. On the other

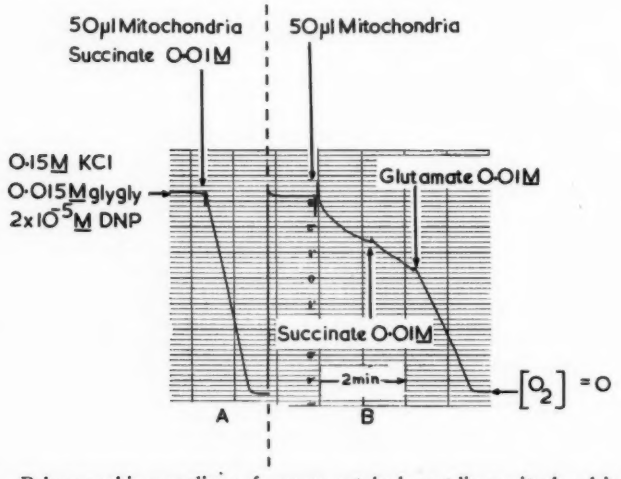

FIG. 1. Polarographic recordings of oxygen uptake by rat liver mitochondria illustrating the marked difference in respiration rates obtained upon addition of succinate simultaneously with the mitochondria or after a brief incubation under the given conditions.

hand, if the mitochondria are allowed to stand in this high potassium, phosphate-free medium in the presence of 2×10^{-5} *M* DNP[3] for a brief period (<2 minutes) before the presentation of succinate, the addition of this substrate then causes almost no acceleration of respiration above the endogenous level, which is quite low by this time. However, glutamate and other pyridine nucleotide-linked substrates can still bring about respiration.

The time course of the inactivation is shown in Fig. 2. The kinetics and precise extent of the process are difficult to determine because of the high endogenous respiration immediately after dilution. It will be seen in Fig. 3 that a spontaneous reactivation of the succinic oxidase system occurs on prolonged incubation. It is instructive to compare Fig. 3 of this paper with Fig. 3 of Ernster and Löw (10).

A number of specific protective and restorative effects have been observed and these are illustrated in Fig. 4, A and B, and Fig. 5. In the first half of Fig. 4 it will be seen that the addition of ATP brings about a reactivation of the succinic oxidase system. This effect is not elicited by GTP, ITP, CTP, UTP, ADP, inorganic phosphate, or versene. The reactivation is preceded by

[3]The following abbreviations are used: DNP, 2,4-dinitrophenol; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; DPNH, reduced diphosphopyridine nucleotide; CSA, cysteine sulphinic acid; tris, tris-(hydroxymethyl)-amino-methane; versene, ethylene-diamine-tetra-acetic acid; Amytal, 5-ethyl-5-isoamylbarbituric acid; and glygly, gylcylglycine.

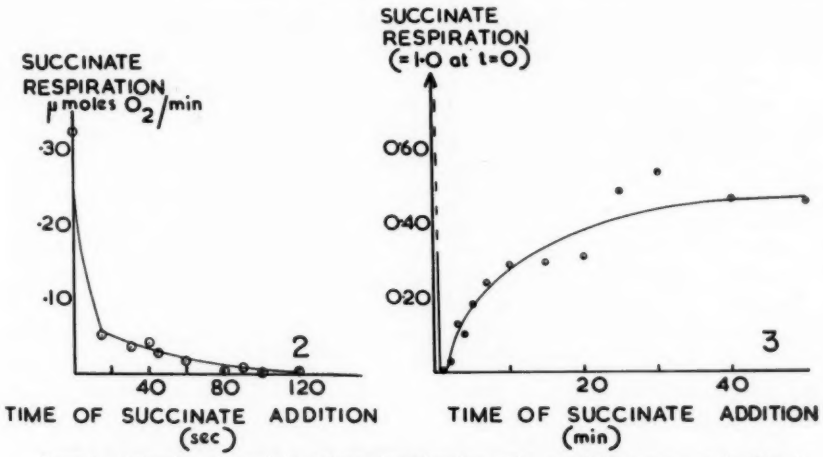

FIG. 2. Kinetics of succinic oxidase inactivation. The conditions were as in Fig. 1 except for the time of addition.

FIG. 3. Spontaneous reactivation of succinic oxidase on prolonged incubation. Similar results were obtained whether the suspension was allowed to stand in the rotating cup of the polarographic assembly or in a slowly rotating Erlenmeyer flask from which 1.0-ml samples were withdrawn for respiration measurements.

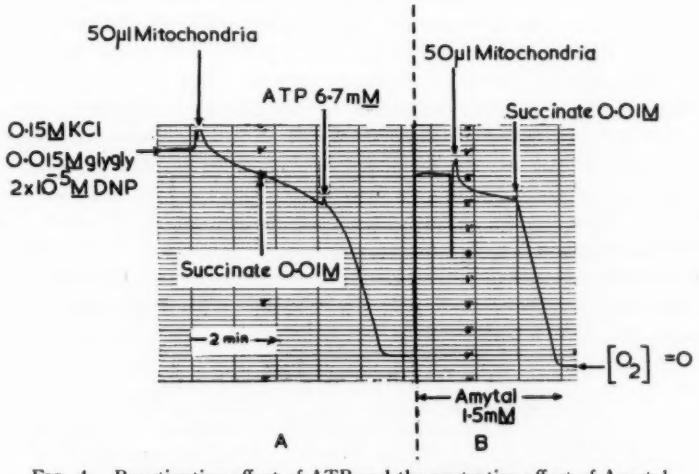

FIG. 4. Reactivating effect of ATP and the protective effect of Amytal.

a short lag phase (see Fig. 4A) and the oxygen uptake continues to accelerate until the termination of the experiment by anaerobiosis.

The second of the specific effects on the inactivation process is shown in Fig. 4B, where it is demonstrated that the presence of 1.5 m*M* Amytal throughout the incubation period provides considerable protection of the succinic

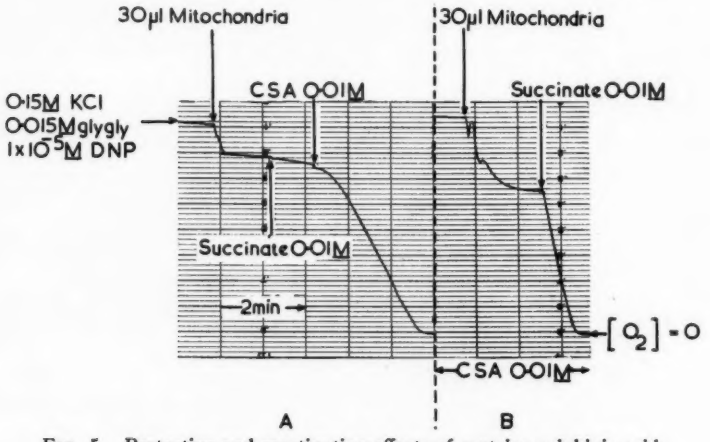

FIG. 5. Protective and reactivating effects of cysteine sulphinic acid.

oxidase activity. Addition of Amytal after inactivation does not reverse the process.

It will be seen in Fig. 5 that the presence of 10 m*M* cysteine sulphinic acid also permits high succinic oxidase activity and that the addition of this compound to the inhibited system partially restores succinate-linked respiration. Other workers have failed to obtain this effect of CSA under rather similar circumstances (11).

The activation of succinic oxidase by ATP is probably significant in connection with the fact that alterations in the medium, to make its composition more like that of media currently in use for the study of oxidative phosphorylation, tend to preserve succinic oxidase activity. Thus, the removal of DNP, the addition of phosphate, the substitution of sucrose of KCl, and the addition of magnesium ions, all gave varying degrees of protection as judged by the extra respiration obtained upon addition of succinate at 90 seconds (Table I). There was considerable variation in the response of different preparations to changes in the ionic constitution of the medium.

TABLE I

Effects of medium on the inactivation of succinic oxidase

Medium	Respiration (µmoles O_2/min)
0.15 *M* KCl, 0.015 *M* glygly, 2×10^{-5} *M* DNP	0.033
Substitute tris for glygly	0.038
Substitute phosphate for glygly	0.165
Substitute sucrose (0.30 *M*) for KCl	0.187
Add Mg^{++} (6.0 m*M*)	0.060
Omit DNP	0.517

NOTE: Fifty microliters of mitochondrial suspension, succinate added 90 seconds after the mitochondria.

Discussion

Similar results to those presented here have very recently been reported (11). As in our original communication (6) the data are explained in terms of the steady-state oxidation–reduction levels of mitochondrial respiratory carriers. However, all of the effects observed in our work are consistent with the simpler hypothesis that the failure of mitochondria preincubated with DNP to respire upon the addition of succinate is due to the inhibition of succinic dehydrogenase by oxaloacetate first described by Das (1). The reactivation by cysteine sulphinic acid is particularly significant since this amino acid rapidly transaminates with oxaloacetate (12). A reactivation of oxaloacetate-inhibited succinic oxidase by ATP was demonstrated by Pardee and Potter (2) and the protective effect of Amytal may be presumed to reflect the inhibition of pyridine nucleotide-linked malic oxidase, since this inhibitor effectively blocks DPNH oxidation (13). An acceleration by barbiturates of the oxidation of succinate by isolated tissues was observed by Jowett and Quastel (14) and the results of these workers may possibly be explained on the basis of a prevention of oxaloacetate accumulation.

Although the effects reported here are consistent with a hypothesis of the inhibition by oxaloacetate of succinate oxidation, the hypothesis lacks direct experimental evidence in the form of oxaloacetate analyses. Since the affinity of oxaloacetate for succinic dehydrogenase is high ($K_i = 1.5\ \mu M$ (2)) and the oxaloacetate may be presumed to arise in the intramitochondrial space, the analytical problem involved may be considerable.

The requirement for an uncoupling agent in order to demonstrate the inhibition of succinic oxidase is of considerable interest since it suggests that the reversal of oxaloacetate inhibition by ATP or other "high-energy" compounds may represent a "normal" mechanism for the maintenance of succinate linked respiration in mitochondria. The physiological implications of such a mechanism for the control of respiration are as yet obscure. One of the principal difficulties in assessing its biological significance is that the chemistry of the mechanism is not established. It would seem at first sight that the oxaloacetic carboxylase reaction leading to the formation of phosphoenolpyruvate (15, 16, 17) would provide such a mechanism but this is not borne out by the nucleotide specificity of the enzyme concerned, oxaloacetic carboxylase having a dual nucleotide specificity for ITP and GTP (18), neither of which are active in the restoration of succinic oxidase activity. Experiments are planned to determine whether there is a "burst" of phosphoenolpyruvate production during ATP reactivation.

The exact relationship of the effects reported here to apparent complexities of the mitochondrial succinic oxidase system reported by others (10, 11) is not clear. However, it is obvious that, although the reactivation of succinate-linked respiration by ATP may involve mechanisms other than that suggested here (6, 11), it is important that adequate experimental evidence be provided that oxaloacetate inhibition is not operative.

Acknowledgments

I am indebted to Dr. C. H. Best for his kind interest in and encouragement of this project, to Dr. Britton Chance for helpful discussions, and to Mrs. Myra Smithies for technical assistance. The investigation was supported by a research grant (A-3523 Nutr.) from the National Institute of Arthritis and Metabolic Disease, United States Public Health Service.

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Addendum

At the first Joint Symposium of the International Union of Biochemists and International Union of Biological Societies on "Biological Structure and Function" (Stockholm, September 1960; proceedings to be published by Academic Press) Ernster and Azzone reported (in distinction to reference 11) that they have observed the "protective" but not the "reactivating" effect of CSA. These authors accept this as evidence for a role of oxaloacetate in the failure of succinate oxidation (although they have been unable to detect it analytically) but suggest that its effect is to bring about, via the oxaloacetic carboxylase reaction, a depletion of high energy intermediates necessary for succinate oxidation rather than to inhibit succinic dehydrogenase. This interesting interpretation is consistent with most of the results reported above although the "reactivation" by CSA (not obtained by Ernster and Azzone) suggests that the hypothesis may not be directly applicable to the phenomena reported here.

A COMPARISON OF PHOSVITINS PREPARED FROM HEN'S SERUM AND FROM HEN'S EGG YOLK[1]

CHI-CHING MOK, W. G. MARTIN, AND R. H. COMMON

Abstract

A phosvitin has been prepared from the serum of estrogenized laying hens and partially characterized on the basis of its contents of N (12.3%), P (10.1%), serine (31.0%), and tryptophan (0.56%). Examination of this serum phosvitin and a yolk phosvitin preparation for N-terminal groups yielded identical chromatographic patterns. Alanine was the major N-terminal amino acid with minor amounts of N-terminal lysine. The sedimentation constant $s^0_{20,w}=3.7$, weight-average molecular weight 4.2×10^4 (Archibald method), and number-average molecular weight 4.0×10^4 (calculated from the tryptophan content) of the serum phosvitin were similar to those of the phosvitin prepared from egg yolk by the same preparative technique. The two preparations behaved similarly when subjected to zone electrophoresis on paper.

Zone electrophoretic evidence is submitted for the presence of phosvitin in the supernatant from dilution precipitation of crude lipophosphoprotein from laying hen's serum as well as in the precipitate itself.

Introduction

It has been known for many years that phosphoprotein appears in the serum of the hen with the onset of laying (1, 2, 3). Brandt *et al.* (4) and Clegg *et al.* (5) studied the differences between the serum proteins of the non-laying hen and of the laying hen by means of free electrophoresis. Clegg and his collaborators (6, 7, 8) have also studied the distribution of phosphorus among the various electrophoretic fractions and have shown that a significant proportion of the phosphorus in the serum of the laying hen was associated with their electrophoretic component 1, which moved in advance of the serum albumin in their experiments, if it be assumed that their component 2 represents the serum albumin of other workers. Schjeide and Urist (9) and Schjeide, Urist, and McLean (10) have conducted ultracentrifugal studies on the proteins of the fowl's serum and egg yolk. Schjeide and Urist (11) considered that their ultracentrifugal entities, serum X_1-phosphoprotein and yolk X_1-phosphoprotein, have a common origin but that they differ in molecular size. They concluded from their own studies and those of others that the "phosvitin" of Mecham and Olcott (12) is a relatively pure preparation of their centrifugally resolved X_1-phosphoprotein of yolk. It is possible by zone electrophoresis in veronal buffer on filter paper (13) to distinguish in egg yolk two lipoprotein zones; α-, β-, and γ-livetin zones; and another zone that corresponds in mobility and staining behavior with phosvitin prepared from egg yolk by the method

[1]Manuscript received June 28, 1960.

Contribution from the Faculty of Agriculture of McGill University, Macdonald College, Macdonald College P.O., Que., and the Division of Applied Biology, National Research Council of Canada, Ottawa. This work was supported by a grant for research from the Quebec Agricultural Research Council.

Issued as Macdonald College Journal Series No. 457 and National Research Council No. 6043.

of Joubert and Cook (14). A similar phosvitin zone was detected on zone electrophoresis of the crude lipophosphoprotein precipitate obtainable from laying hen's serum by dilution precipitation. This zone ran faster than serum albumin under the given conditions.

These observations suggested that it should be possible to prepare a phosvitin from avian serum as well as from egg yolk by a suitable biochemical procedure. The present paper describes such a preparation and a comparison of its properties with those of phosvitin prepared from egg yolk by the same method. A phosphoprotein detected in avian serum by ultracentrifugation had been found to have a high content of serine (9, 10, 11), and a preliminary account of the present work (15) reported the preparation and partial characterization of phosvitin from hen's serum. This phosvitin is evidently either derived from or identical with the phosphoprotein previously observed by Schjeide and Urist (9, 10, 11). The present paper presents more detailed information on the preparation of phosvitin from serum and its partial characterization.

Experimental

The Preparation of Phosvitin from Hen's Serum

Initial attempts to recover phosvitin from the serum of laying hens by the method of Joubert and Cook (14) were inconclusive even though as much as 400 ml serum was used in one experiment. In several experiments small yields of presumptive phosvitin were obtained, but these were insufficient for characterization. It was thought that these inconclusive results might be due to a relatively low level of phosphoprotein in the sera of hens in full lay, for the level of lipophosphoproteins in hens in full lay may be much lower than it is just before laying begins (16). Accordingly, it was decided to repeat the experiments with serum from hens in which the level of serum lipophosphoproteins had been enhanced by treatment with estrogen.

Four laying hens (White Plymouth Rock) were each given 2 mg estradiol benzoate (as 'Progynon B' Schering) intramuscularly for 5 successive days. Laying was not interrupted by this treatment. The hens were killed and bled and 200 ml serum was recovered. This serum was adjusted to pH 5.3 by slow addition of 80 ml 0.1 *N* HCl with constant stirring. Distilled water was then added slowly to this slightly acidified serum until the total volume reached 400 ml. The mixture was left to stand overnight at 6° C. Next morning the precipitate of crude lipovitellin complex was separated on the centrifuge (3000 r.p.m. for 30 minutes), redissolved in 1% NaCl, and reprecipitated by 10-fold dilution with distilled water.

The precipitate was taken up in 25 ml of 0.4 *M* $MgSO_4 \cdot 7H_2O$ and sufficient of the solid salt was added to provide a final concentration of 0.4 *M* $MgSO_4$. The resultant clear solution was diluted with an equal volume of distilled water. The precipitate of crude lipophosphoprotein was separated on the centrifuge, taken up again in 0.4 *M* $MgSO_4$, and reprecipitated by dilution as

before. All the supernatants to this stage were discarded.

The partially purified lipophosphoprotein material was dissolved in 10% NaCl and centrifuged at the highest speed possible (approx. 4400 r.p.m.) in an International refrigerated centrifuge Model PRI. Lipid material separated at the surface and was removed by filtration through a wad of packed cheesecloth. The filtrate was diluted with twice its volume of water and then dialyzed against several changes of acetate buffer (pH = 4.0, $\mu = 0.1$). This resulted in the formation of a precipitate considered to be crude lipovitellin.

The water-clear supernatant was separated from the precipitate by centrifugation and was dialyzed against several changes of distilled water to remove the buffer. The solution remained clear. It was then lyophilized and the fluffy pure white product was dried to constant weight *in vacuo* over concentrated H_2SO_4. The final yield from 200 ml serum was 150 mg phosvitin.

Chemical Composition of Serum Phosvitin

The phosvitin was analyzed for nitrogen by micro-Kjeldahl and for phosphorus by the method of Berenblum and Chain (17). Tryptophan was determined by the method of Shaw and McFarlane (18). Serine was determined by Boyd's modification of the method of Shinn and Nicolet as described by Block and Bolling (19). Phosphate was removed from ester linkages by incubation with 1% NaOH before hydrolysis (12). The values for serine were corrected for a presumed loss of 10% of serine during hydrolysis. Check determinations of known amounts of serine that had been submitted to the same hydrolytic procedure as had the phosvitin indicated a loss of approximately 10%, a loss similar to that reported by Mecham and Olcott (12). The analytical values obtained for serum phosvitin and yolk phosvitin are presented in Table I, together with the values reported by Mecham and Olcott (12) for phosvitin prepared by their method.

TABLE I

Analysis of phosvitin prepared from hen's serum and from egg yolk by the method of Joubert and Cook (14), together with analyses reported by Mecham and Olcott (12), all values in %

	Serum phosvitin	Yolk phosvitin	Yolk phosvitin (12)
N	12.3	12.9	11.9
P	10.1	10.4	9.7
Serine	31.0	31.0	31.3
Tryptophan	0.56	0.58	0.6

The values for serum phosvitin and yolk phosvitin were closely similar and were in reasonable agreement with those reported by Mecham and Olcott (12) for yolk phosvitin. This suggests strongly that the phosvitin from serum had the same composition as that from egg yolk.

The yield corresponded to approximately 7.5 mg phosvitin P per 100 ml serum. This value lies within the range of values for phosphoprotein P found

in the sera of pullets that had received roughly similar treatments with estrogen, but it is unlikely (see below) that the recovery of phosvitin was even approximately quantitative.

Examination of Serum Phosvitin for N-terminal Amino Acids

Neelin and Cook (20) have reported that the major N-terminal amino acid of egg yolk phosvitin is alanine. Accordingly, samples (40 mg) of the serum phosvitin and of egg yolk phosvitin preparations were examined for N-terminal amino acids. The treatment with 1-fluoro-2,4-dinitrobenzene and hydrolysis were done as described by Fraenkel-Conrat *et al.* (21). Separation of N-2,4-dinitrophenyl (DNP) derivatives was done as described by Blackburn and Lowther (22). The first run in the two-dimensional chromatography of DNP derivatives was made with *tertiary* amyl alcohol saturated with the buffer that was used to saturate the paper (22). The second dimensional run was made with concentrated phosphate buffer as described by Levy (23).

The resultant chromatographic patterns are shown in Fig. 1. It will be seen that the patterns for the two phosvitins are well-nigh identical. The predominant N-terminal amino acid of both phosvitin preparations was alanine (see Fig. 1, spot 1), in agreement with the report of Neelin and Cook (20) on the end groups of egg yolk phosvitin.

A second DNP-amino acid (Fig. 1, spot 4) was present on both chromatograms in minor amounts relative to DNP-alanine. Neelin (24) had also observed the presence of a spot corresponding to spot 4 (Fig. 1) on chromatograms of DNP derivatives from yolk phosvitin and had concluded that it represented a minor but significant proportion of N-terminal lysine. Accordingly, it became desirable to confirm whether spot 4 represented bis-DNP-lysine, since bis-DNP-tyrosine appears in the same region as bis-DNP-lysine on such chromatograms. Separate portions, (*a*) and (*b*), of a hydrolyzate of DNP-phosvitin (serum) were chromatographed after addition of bis-DNP-lysine to portion (*a*) and of bis-DNP-tyrosine to portion (*b*). The former chromatogram yielded one spot in the approximate region of spot 4, the latter yielded two spots in this region. It was concluded that spot 4, Fig. 1, represented bis-DNP-lysine.

The foregoing evidence from the determinations of N-terminal amino acids supported strongly the view that the two phosvitin preparations were closely similar, if not identical.

Sedimentation Coefficient and Mean Molecular Weight of Serum Phosvitin

Sedimentation coefficients of serum phosvitin in 0.5 *M* NaCl were obtained from measurements made in the Spinco ultracentrifuge in 30-mm cells at 50,740 r.p.m. Extrapolation to zero concentration (Fig. 2A) resulted in $s^0_{20,w} = 3.17 \pm 0.05$, in good accordance with $s^0_{20,w} = 3.14$ reported for egg yolk phosvitin in 0.5 *M* NaCl by Joubert and Cook (14). The partial specific volume of serum phosvitin was assumed to be the same as that determined for yolk phosvitin, viz., 0.545 (14). A typical sedimentation velocity pattern

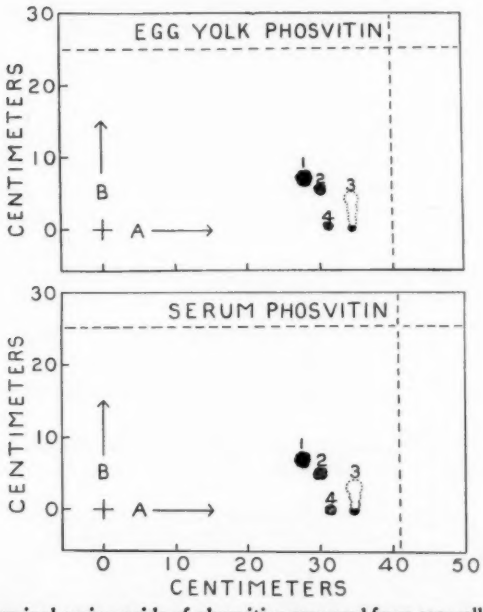

FIG. 1. N-Terminal amino acids of phosvitins prepared from egg yolk and from serum.
(A) Run in *tertiary* amyl alcohol.
(B) Run in 1.5 *M* phosphate buffer, pH 6.0. Broken lines indicate positions of solvent fronts. Cross indicates point of application.
Spot 1. DNP-alanine. Spot 3. 2,4-Dinitroaniline.
Spot 2. 2,4-Dinitrophenol. Spot 4. bis-DNP-lysine.

obtained from operation with two cells in the rotor is shown in Fig. 3A.

The molecular weight of serum phosvitin in 0.5 *M* NaCl was estimated on the basis of Archibald experiments (25) performed at 17,980 r.p.m. at three initial solute concentrations (c_0). Schlieren patterns from these experiments are shown in Fig. 3, B to F. A Spinco synthetic boundary cell (26) was used to obtain c_0 from patterns similar to Fig. 3, B and C. Fig. 3, D, E, and F, are illustrative of the patterns used to obtain molecular weights from Archibald measurements. Base lines were flat as shown by separate solvent experiments under similar conditions to those of the Archibald experiments. It is also evident from Fig. 3, B and C, that the base line at the meniscus was flat. Although Kel F polymer oil No. 1 (27) was added to the cells to form a lower interface, measurements were based on the intersection of the Schlieren pattern with the air-solution interface only. Measurements at the base of the cell (oil–solution interface) were not made because of the difficulty of extrapolating the Schlieren pattern. Computations were made by the procedure of Trautman (28) and Smith *et al.* (29) to obtain the apparent molecular weights (M_{app}), which were then plotted, as recommended (30), against the square root of the elapsed sedimentation time ($t^{\frac{1}{2}}$), as shown in Fig. 2B. The M_{app}

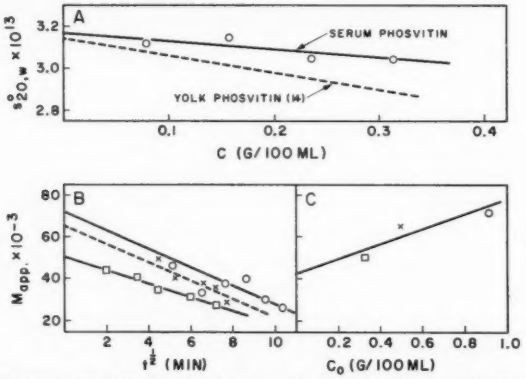

FIG. 2. Sedimentation behavior and molecular weight of serum phosvitin (solvent 0.5 *M* NaCl).
(A) Sedimentation.
(B) Apparent molecular weight–time relation at three initial concentrations.
(C) Apparent molecular weight–concentration relation at zero time.

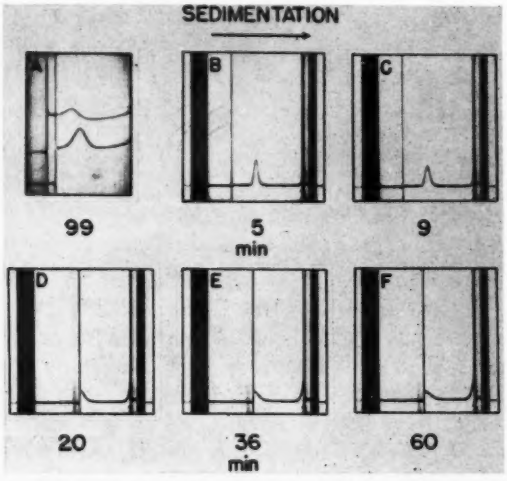

FIG. 3. Ultracentrifugal patterns of serum phosvitin in 0.5 *M* NaCl solution.
(A) 50,740 r.p.m.; c_0=0.31 and 0.16 g/100 ml. Schlieren diaphragm 70°; 30-mm cells.
(B) 17,980 r.p.m.; c_0=0.50 g/100 ml. Schlieren diaphragm 80°; synthetic boundary cell.

derived by extrapolation to zero elapsed time were then plotted versus each corresponding value of c_0, and extrapolated to obtain the weight-average molecular weight at infinite dilution (Fig. 2C). This gave a value of 4.2×10^4. Considering the magnitude of the molecular weight and the different methods employed, this value is in good agreement with the correct value for egg yolk

phosvitin (3.6×10^4) based on the measurements of Joubert and Cook (14)*.

The negative slope of the lines in Fig. 2B suggests polydispersibility, since M_{app} decreases with time. Since the concentration at the meniscus falls with time, concentration dependence alone should yield curves of positive slope, and an ideal monodisperse system should yield a constant M_{app}. There is evidence of concentration dependence in Fig. 2C but M_{app} increases rather than decreases with concentration. A similar effect has been observed in polydisperse systems below the Flory temperature (32). This suggests that phosvitin may become polydisperse by a tendency to aggregate as the concentration increases. Further ultracentrifugal studies were precluded owing to the limited amount of the serum phosvitin that was available.

The chemical analyses also provide some information as to number-average molecular weight. If it be assumed that the serum phosvitin molecule contains one only tryptophan residue, then the tryptophan content of 0.56% would indicate a number-average molecular weight of 3.95×10^4. This figure is similar to the value obtained by the ultracentrifugal studies, and suggests that the phosvitin molecule does in fact contain but one tryptophan unit. Again, if it be assumed that all of the phosphorus (10.1%) is bound in phosphoserine units, then the molecule must contain 52.9% of phosphoserine residues. If it be assumed that the molecular weight is 4.0×10^4, then 53.9% phosphoserine residues correspond to 129 phosphoserine units per molecule. The main importance of the foregoing observations on particle size is, however, the substantial identity of all the values obtained by ultracentrifugal methods for the phosvitin prepared from serum with those obtained for the phosvitin prepared from egg yolk.

Zone Electrophoretic Behavior of Serum Phosvitin

A sample of serum phosvitin was subjected to filter paper electrophoresis in veronal buffer (pH 8.6, $\mu=0.05$) as described by McCully *et al.* (13) and a sample of phosvitin prepared from egg yolk was run concurrently on the same strip. No difference in electrophoretic mobility was detected. Both preparations exhibited a similar ready staining with Amidoschwarz 10B and a similar tendency for the dyestuff to be washed out again when the strips were washed to remove unbound dyestuff. Neither preparation showed any trace of lipid staining with oil red O.

McCully *et al.* (13) have shown that a zone corresponding in mobility and staining properties with phosvitin may be observed on filter paper electropherograms of crude serum lipophosphoprotein obtained by dilution precipitation from serum of hens. No such zone was seen on similar electropherograms of unmodified serum from laying or estrogenized hens.

When the supernatant from dilution precipitation of lipophosphoprotein was submitted to zone electrophoresis in agar by the method of Giri (33), a

*Through a computation error the diffusion coefficient reported earlier (14) was too high and M therefore too low. The correct values of egg yolk phosvitin in 0.5 *M* NaCl are $D=4.6\times10^7$ and $M=3.6\times10^4$ (31).

phosvitin zone was observed just in advance of the albumin zone. This showed that only a fraction of the phosvitin is thrown down with the crude lipophosphoprotein. It also suggests that part at least of the phosphoprotein P in the supernatant from precipitation of 'lipophosphoprotein complex' described by McIndoe (34) may be phosvitin.

Reproducibility of Isolation of Serum Phosvitin

It was considered advisable to examine the reproducibility of the isolation of serum phosvitin described above. The entire procedure was repeated on four different lots of serum with results that are summarized in Table II.

TABLE II

Isolation of serum phosvitin from sera of estrogenized laying hens

Experiment No.	No. birds used	Treatment estrogen	Vol. of serum, ml	Total serum phosphoprotein P as mg/100 ml serum	% of total serum protein P recovered as phosvitin P
1	5	2 mg ODB per day for 5 days	185	20	2.3
2	6	2 mg ODB per day for 5 days	250	18	6.2
3	48	0.5 mg ODB per day for 15 days	720	13	8.5
4	12	2 mg ODB per day followed by 4 mg stillbestrol per day for 3 days	360	25	16.6

The proportions of the total serum phosphoprotein P obtained as purified phosvitin P were low and varied greatly as between the four different experiments. The low recoveries were in part due to the obvious circumstance that not all of the serum phosvitin is precipitated with the crude lipophosphoprotein complex (see above). Again, lipovitellin itself contains around 0.5% P on a lipid-free basis (14), which would depress the recovery of total serum protein P as phosvitin P. It would seem, nonetheless, that it should be possible to devise a biochemical technique that would permit the recovery of a much higher proportion of the total serum phosphoprotein P as phosvitin P.

Acknowledgments

The authors wish to thank the Quebec Agricultural Research Council for the grant which defrayed part of the costs of this work and provided a research assistantship for the first author. They are indebted to Dr. W. H. Cook, Division of Applied Biology, National Research Council of Canada, for much helpful advice and criticism. They wish also to thank the Schering Corporation, Montreal, for generous gifts of hormonal preparations.

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QUANTITATIVE CHROMATOGRAPHIC METHODS

PART 2. AN APPROACH TO PAPER CHROMATOGRAPHY OF IMPROVED RESOLVING POWER AND REPRODUCIBILITY[1]

CHARLES S. HANES

Abstract

The problems involved in developing an improved and more comprehensive system of analysis of amino acids and peptides based on paper chromatography are considered broadly and the scope of a group of investigations directed to this objective is described in outline. In addition to improvement and simplification of analytical techniques, these studies are to be concerned with an attempt to secure substantial improvement in the resolving power and reproducibility of the one-dimensional paper chromatogram. An important contribution to this objective has come from modifications in the chromatographic equipment designed to provide closer control of the physical conditions under which the filter paper is irrigated with the chromatographic solvent. The reasons for these modifications in the design of equipment are described. Other topics dealt with in this introductory paper relate to the purity of chromatographic papers, procedures for their pretreatment with buffer solutions, and some useful ancillary equipment which has been developed.

General Introduction

The Objective

In an earlier paper by Connell, Dixon, and Hanes (1) — now considered to constitute Part 1 of this series of communications — a method was described for the quantitative study of enzymic transpeptidation reactions based upon the determination of peptides and amino acids separated by one-dimensional paper chromatography. The areas of paper or 'spots' occupied by ninhydrin-reacting compounds were excised, treated to eliminate ammonia, and then heated with a quantitative ninhydrin–hydrindantin reagent to obtain full development of the Ruhemann's purple. The color was then eluted for measurement, and the absorbancy corrected for a small amount of color given by an appropriate 'paper blank'. Extensive application of this analytical method to various transpeptidation systems to be described shortly has established its general reliability for the determination of peptides and amino acids, provided that these can be separated as compact individual spots on the chromatograms; subsequent modifications have led to simplification and improved accuracy of the analytical procedures.

In the enzymic studies mentioned the chromatographic separations presented little difficulty since not more than five or six peptides and amino acids were normally present in the reaction mixtures, and substrates could be selected with some regard to chromatographic properties. However, the need for more refined chromatography became apparent when it was desired later to apply the analytical procedure to more complex enzymic systems and to other

[1]Manuscript received June 27, 1960.
Contribution from the Department of Biochemistry, University of Toronto, Toronto, Ontario.

problems involving the analysis of amino acids present in complex mixtures, as in various biological fluids and hydrolyzates of proteins and peptides. Despite the merits for some purposes of various two-dimensional procedures, either two-dimensional chromatography (2, 3, 4) or electrophoresis in one direction and chromatography in another (5, 6, 7, 8), it was desirable for reasons which will be made clear to continue, if possible, to use one-dimensional descending chromatography as the basis of the quantitative method. This necessitated an attempt to develop a small set of complementary chromatographic systems (preferably two or at most three systems) in one or other of which each amino acid could be separated as a compact and analyzable spot.

Despite the wealth of published information relating to the chromatography of the amino acids, the development of something approaching an ideal complementary set of one-dimensional systems has been a formidable task. The success which has been attained is the result of a succession of innovations introduced during a period of several years; in their cumulative effect these may be claimed to have resulted in great improvements in both resolving power and reproducibility of the one-dimensional paper chromatogram. Improved separations of the amino acids are now attainable easily under conditions which are clearly defined; they are but little affected by the presence in the solutions to be analyzed of salts in concentrations as high as 0.5 molar (or even 1 molar in some cases) or by wide variations in either the total concentration or the relative proportions of the amino acids present. Such improved chromatography has been found to provide a serviceable basis for the separation and quantitative determination of the amino acids in connection with a variety of problems. Of wider interest is the fact that the approach which has been effective in securing superior resolutions of the amino acids promises to be applicable also to improving the separation of the other groups of compounds. For example, parallel studies have led to improved separations of the nucleotides. There are indications that systems of enhanced resolving power can be developed also for peptides.

The Practical Approach and a View of the Mechanism

Puckishness in a chromatographic system — often accepted as an inevitable weakness of paper chromatography — reflects the sensitivity of the particular system to variations in unrecognized or ill-defined factors. A practical approach which has led gradually to much improved chromatography has rested largely upon the study and exploitation of such sensitivity; essentially it has been to identify successive factors which influence the pattern of separation and to adjust these factors in turn in an attempt to secure desired resolutions. In this sense puckishness and sensitivity may be taken to indicate the adjustability of a system, and hence its potentialities for controlled modification and improvement.

In following any such empirical approach to the refinement of chromatographic systems, it is the common experience of investigators to encounter clear,

reproducible effects for which no rational explanation can be offered in terms of currently formulated concepts of the mechanism of the paper chromatogram. In fact, such formulations tend to remain so general as to offer little guidance at the operational level, despite the fact that numerous studies have dealt with particular aspects of the physical system, such as the structure of filter paper (9, 10, 11, 12), liquid flow within the void spaces of filter paper (13, 14, 15, 16), the nature of the stationary phase on the paper chromatogram (2, 17, 18, 19), the existence of heterogeneity in separational properties on the chromatogram (2, 20, 21, 22), and numerous individual factors influencing the migration rates of particular solutes in various systems (cf. reviews in 16, 23, 24).

A simple theoretical picture of the functioning paper chromatogram which appears to be accepted widely may be described briefly. The two essential components of the actual separatory mechanism are the moving liquid phase, which is drawn progressively into the void spaces of the filter paper by the forces of capillary attraction (modified to a slight degree by gravity), and the stationary phase, which is formed upon the cellulose elements of the paper and which is envisaged as some form of cellulose–water–solvent complex such as a gel, with or without associated 'free' liquid. Solutes present in this dynamic two-phase structure are transported at rates which reflect their specific partitioning between the moving and stationary phases. In paper chromatography (unlike column chromatography) the two functional phases are surrounded by an extensive vapor phase which occupies the main volume within the sealed chromatographic unit. Usually an additional fourth phase is present in the closed system, namely a pool of a 'conditioning solvent', which is included with the intention, rarely achieved, of 'pre-equilibrating' vapor phase and filter paper with the vapors from a liquid of the same composition as the chromatographic solvent, to be added later.

It requires no emphasis that the detailed characteristics and the practical usefulness of the separations obtained on the finished chromatogram are determined by more subtle features of the functioning system than are taken into account in this basic mechanistic picture. The probable nature of some of these features is suggested by various effects which have been established in the studies to be described below, often in confirmation of observations by other writers. For example, it has been found that the rate of supply of solvent to the chromatogram may influence the shape and size of the separated 'spots', the permissible load of amino acids, and their relative linear spacing along a particular pattern of separations. Again it has been established that different batches of filter paper may show characteristic differences in the linear extension of particular regions of a given pattern of separations, due apparently to differences in the porous structure of the filter paper. Analogous expansions or contractions of regions of a pattern may be induced by minor changes in solvent composition of which the main effect would seem to be upon viscosity. The interpretation of these effects, and of a range of other effects which result obviously from the occurrence of progressive net transfers of constituents

between the different phases of the system during the process of irrigation, requires clearly a more detailed and comprehensive understanding of the 'separational field' or, more probably, the sequence of such fields, to which the solutes become subjected during their migration down the chromatogram. Over any small area of the chromatogram the separational field (which might be defined empirically in terms of the rates of migration of specified solutes) may be assumed to be determined by such properties as: (*a*) the compositions and volumes per unit area of the moving and stationary phases, which would determine the equilibrium distribution of a given solute between these phases; (*b*) the geometrical interrelation between these phases (e.g. their effective thicknesses and the area of interface between them) which would influence the rates of diffusion of solutes from one phase to the other and perhaps the extent of spreading by diffusion in either phase; and (*c*) the effective flow rate of the moving liquid phase over the functional interface, which will determine the maximum rate of transport.

Even this rudimentary designation of the properties which may be the main determinants of the local separational fields on the chromatogram permits plausible extensions of the simple theoretical picture of the chromatogram and, in turn, plausible interpretations of various effects of operational importance. Perhaps of central importance has been the realization that under no conditions normally attainable can the paper chromatogram be assumed to possess a uniform separational field over the whole of its functioning area. Consideration shows that all the underlying determinants of the local separational field are liable to variations from a variety of causes. For example, the progressive loss of one or more constituents from the flowing liquid phase into the vapor phase (or the reverse process) will result in progressive alterations along the invaded portion of the chromatogram in all or most of these determinants. This will be true also if there occurs net transfer of constituents between the liquid phase and the stationary phase. Whilst such transfers may be reduced or modified by some form of preconditioning of the system, this is probably never fully effective in practice, and it is ineffective so far as any non-volatile constituents added to either the solvent or the paper are concerned. Furthermore, as will be seen, the inherent heterogeneity of the capillary void spaces of the filter paper constitutes in itself an additional cause for the longitudinal variation in the separational field on the chromatogram. This arises from the fact that near the solvent source, substantially the whole of the void space, including all categories of capillary channel, will be occupied by the flowing liquid phase, whereas near the advancing front (where the impedance to supply of solvent will be greatest) the liquid will be drawn into only the finest capillary channels which exert the maximum suction force upon it; at intermediate levels between these extreme positions, intermediate fractions of the void space (and intermediate categories of capillary channel) will be occupied by the liquid phase. It is evident that the separational field may be extremely different at different levels of the chromatogram as a result of this intricate process of penetration.

Not only will the volume of the liquid phase and its geometrical relationship with the stationary phase vary greatly along the chromatogram, but the variation in the range of channel widths occupied at different levels is likely to result in complex variations in the linear flow rates in different portions of the advancing liquid continuum; for example, at levels at which wide categories of capillary channels are occupied by the liquid, the flow through narrow categories of channel may be largely bypassed owing to the higher resistance they will offer to viscous flow. If narrow channels provide the bulk of the functional separatory mechanism, as may well be the case, then the effective flow rate may be reduced greatly from this cause. This brief consideration of the process of irrigation of the chromatogram suggests that the longitudinal profile of the liquid phase (e.g. volume per unit area and effective flow rate / distance from solvent source) and the resulting gradient in the separational field will reflect, on the one hand, the distribution of channel width characterizing the porous structure of the particular filter paper and, on the other hand, the physical properties of the solvent, notably viscosity and surface tension.

The broad conception emerging from the considerations touched upon here is that the functioning chromatogram is so intricate a physical system that many of its basic properties remain uncharted in any quantitative sense. The complexities arise in part from the inherent heterogeneity of the filter paper structure and in part from progressive interactions between the different gross phases in the system during the process of irrigation. The longitudinal gradients of the separational field on the chromatogram must be pictured as an inherent feature which can be modified but not eliminated by the usual measures adopted for 'preconditioning' the system.

It must be assumed that, in general, when two solutes become separated on a chromatogram, they will pass into significantly different separational fields characterizing their new positions. It follows that each separated solute or group of solutes observed on the final chromatogram will have passed through its own distinctive sequence of separational fields in the course of its migration from the point of origin. It is likely, in fact, that the remarkably high resolving powers exhibited by some systems under particular conditions may depend upon the existence of a heterogenous separational field such that separations which are not effected at one level may occur at another. The realization of this possibility has led, as will be illustrated in the ensuing studies, to successful attempts to modify and to accentuate longitudinal variations in the separational field in particular systems as a means of securing desired separations and adjustments of the pattern of separations.

The Scope of the Investigation

Apart from the general considerations already touched upon, the present paper (Part 2 of the series) is to deal with modifications in the equipment for descending paper chromatography which have resulted in closer control and improved effectiveness of many systems and which have added both to the

convenience of operation and to the protection of operators from toxic solvent fumes.

Parts 3 and 4 will describe the development of new chromatographic systems of high resolving power for the amino acids. Part 3 will present the results of a survey of the separations obtained in various types of chromatographic system and of the manner in which a variety of factors influence the separations in some selected systems, especially buffered water-miscible solvents used with buffered papers, while Part 4 will be devoted to the description of a selected small number of these systems under conditions adjusted to yield the most desirable patterns so far attainable.

Parts 5, 6, and 7 of the series will deal with the improvement and simplification of the actual analytical determinations of amino compounds separated on the chromatograms. For example, in Part 5 a modified ninhydrin–hydrindantin reagent of improved stability and accuracy will be described, while Part 6 will deal with a simplified procedure for the assay of amino acids in the excised paper 'spots' from chromatograms. Part 7 will be concerned with a procedure for isolating amino acids from crude biological fluids, such as serum, and preparing solutions of them suitable for quantitative chromatography.

In Part 8 a selection of chromatographic systems developed for the quantitative study of nucleotides will be described.

Equipment for Improved Paper Chromatography

Constant Temperature Cabinet and Vapor Hood

The maintenance of a constant temperature is desirable during the irrigation of paper chromatograms since it may contribute greatly to the reproducibility of the separations obtainable in most systems. Systems differ widely in sensitivity to temperature variations as is to be expected from the diversity of possible effects, e.g. upon the viscosity and surface tension of the solvent, upon the evaporation of its constituents, and upon the distribution both of solvent constituents and of the solutes under study between the moving and stationary phases on the paper. After trials of temperatures ranging from 20° C to 40° C, an operating temperature of 34° C was adopted in this work; this temperature appeared nearly optimum for some, and suitable for all, of the systems which have been used, and it can be maintained in most climates without providing cooling.

In planning facilities for chromatography at a constant temperature, attention should be given to the protection of operators from the toxic vapors of solvents. The housing of the chromatographic units in a small closed room at a constant temperature does not usually lend itself to adequate ventilation. Both temperature control and ventilation are attainable economically by an arrangement illustrated in Fig. 1, which was developed in association with Dr. F. A. Isherwood* and which has been in use in a number of laboratories since 1948.

*of the Low Temperature Station for Research in Biochemistry and Biophysics, Cambridge, England.

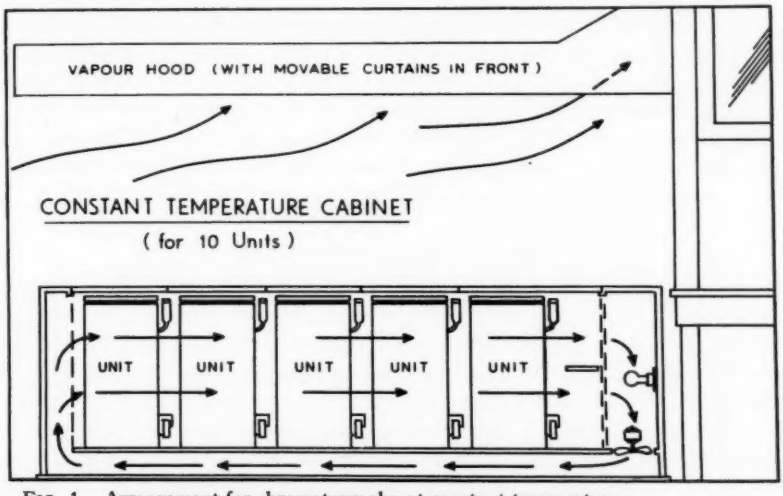

FIG. 1. Arrangement for chromatography at constant temperature.
The constant temperature cabinet, with insulated walls and internal air circulation, stands under a vapor hood provided with movable plastic curtains on tracking along its front edge (not shown). The cabinet illustrated (with front removed) holds five pairs of chromatographic units and has an over-all length of 128 in. Shelf at right is convenient for storage of solvent at temperature of operation.

A simple form of constant temperature cabinet, holding the desired number of chromatographic units (tanks or boxes), is placed along a wall under a vapor hood, preferably adjacent to a large fume cupboard in which various operations connected with the chromatography are conducted.

The constant temperature cabinet is of bench height, and has removable lids which give access to the actual chromatographic units, which are arranged in pairs inside. The units rest on a platform which has a duct space beneath it. Air is drawn along the main upper chamber by a fan at one end and is recirculated via the lower duct, passing over a thermoregulator and a heater (two 100-w lamps) at appropriate points in its path. The temperature is maintained to within 0.5° C except for the occasional short periods when the lids of the cabinet are removed for access to the units.

An overhead vapor hood, with exhaust preferably at one end, extends over the length of the cabinet; this carries curtains, on tracking along the front edge, to form a closed passage over the cabinet along which a current of air is drawn from its open end to the exhaust. By sliding aside the appropriate curtain all necessary operations can be carried out with negligible inhalation of vapor, and with no contamination of the general atmosphere of the laboratory.

Other pieces of equipment may be included in this arrangement to form a compact chromatographic bay at one end of a laboratory. Simple racks are provided under the vapor hood for suspending wet chromatograms to dry in the air stream. An illuminated panel, forming part of the back wall of the fume

cabinet is useful as a background for spraying or examining chromatograms. A warm air oven (e.g. for the development of color reactions on chromatograms) is housed conveniently under the vapor hood. For drying chromatograms which have been irrigated in phenol, a tall chamber, through which a current of filtered warm air at 40° C is blown, may be set into the floor of the fume cupboard. Finally, a 'spotting' bench completes the general equipment of a convenient chromatographic bay.

The Chromatographic Unit

Since the original work of Consden, Gordon, and Martin (2) the typical unit of equipment for descending chromatography has consisted essentially of a more or less vapor-tight container (jar, tank, or box) with suitable supports inside for a solvent trough and for the chromatograms which are to be irrigated with solvent added to the trough. The unit contains usually, in addition, some means, however simple, for modifying or 'conditioning' the atmosphere within the unit. There might seem to be little scope for innovations in these arrangements which could result in major improvements in chromatography. In fact, however, successive modifications in the components have resulted in a series of units which have given progressively improved chromatograms.

Only two designs of unit; to be referred to as the type 3 and type 4 units, require specific mention. The type 3 unit was used in most of the studies to be described in Part 3 of this series, on the influence of variations of different factors upon the patterns of separation. On this basis was developed the final type 4 unit, which affords simple and effective means for adjusting and controlling various factors now recognized as important for reproducible operation of chromatographic systems of high resolving power.* The operation of the selected systems to be described in Part 4 of this series will be specified in terms of the type 4 unit. The types 3 and 4 units are illustrated in Figs. 2 and 3 respectively. The design of the components of the units in relation to function requires separate discussion.

(a) The Box and Its Closure

The investigation was begun with an all-glass chromatographic unit based upon an earlier design (17) but fitted with an electromagnetic fanning device for bringing about equilibration with a large pool of solvent in the bottom of the glass jar. When the use of new systems and conditions brought about improved chromatography, particularly smaller and more nearly circular spots of the faster-running amino acids, it became advantageous to increase the length of the chromatograms beyond that which could be accommodated in any available glass jars. (The present total length of the chromatogram as mounted in the unit is ca. 31 inches, of which 24 inches is available for the travel of solutes under study; see below.) Accordingly larger non-glass containers constructed

*Chromatographic units of the final type 4 design are being manufactured by New Way Laminates Ltd., 29 Portland Street, Mimico, Toronto (14). These have been found satisfactory in all respects.

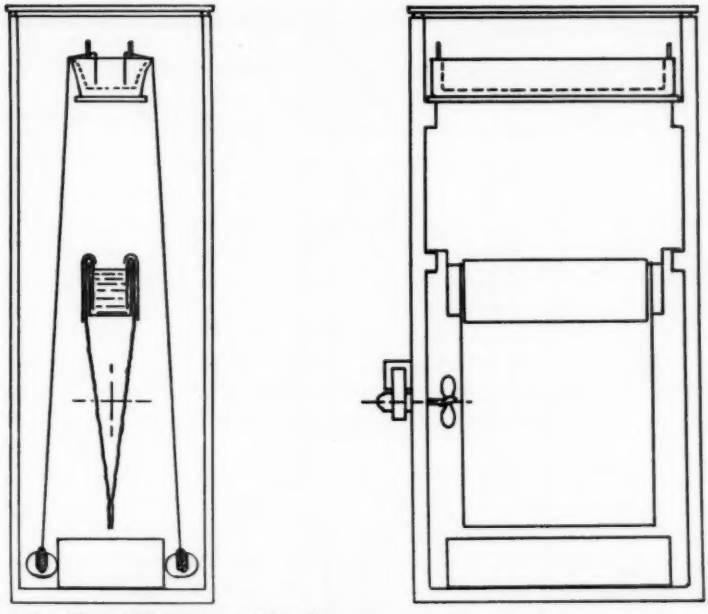

FIG. 2. Type 3 chromatographic unit.
Drawings show solvent trough, equilibrating system, and (in left section only) two chromatograms mounted in place in the unit.
A main feature of the design was the efficient system for equilibration with any selected 'conditioning solvent' placed in reservoir in middle. Note evaporating curtains of No. 3 paper, drip tray below, and fan to circulate air over the wet curtains during period of preconditioning. Studies with this design led to the final type 4 unit shown in Fig. 3.

of various materials were tested, e.g. metal, glass in wooden frames, sheet plastics, wood coated with different synthetic varnishes, and finally, for the type 4 unit, a complex laminated board, faced inside with formica sheeting (containing an embedded metal foil barrier) and faced outside with a sheet of melamine polymer.

In the type 3 unit, plywood boxes coated inside and out with a so-called 'epoxy resin' varnish were used for several years with moderate success. However, when it was found desirable to eliminate entirely any internal pool of liquid as a means of conditioning the closed system (but instead to add small measured volumes of desired volatile constituents) it was found that the 'epoxy resin' coating was too permeable to vapors to meet the more exacting requirements of this new method of operation.

The type 4 unit was then developed, constructed of the laminated material described above; this has proved to be eminently satisfactory from all points of view. The walls appear to be virtually impermeable and there has been no sign of alteration in the course of many months of constant use under the varied

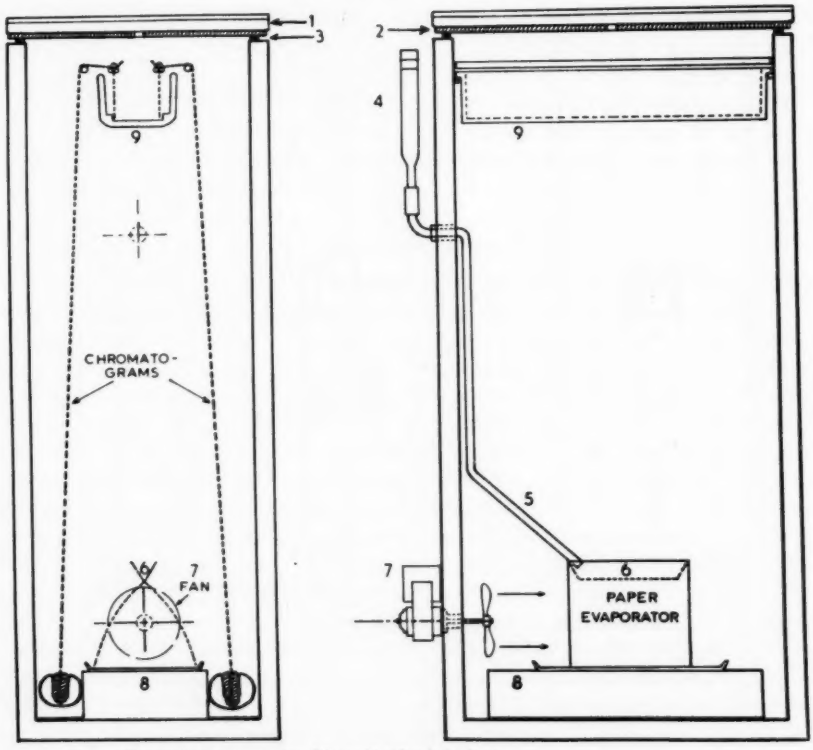

FIG. 3. Type 4 chromatographic unit (final design).
Diagram shows solvent trough and (in left section only) two chromatograms mounted in place in the unit, with feeder wicks in the trough, absorption pads secured by tubular clamps at bottom. Inside dimensions of the box at 13 in. × 9¼ in. × 28¼ in. high. (1) weighted cover; (2) glass lid, with central hole; (3) neoprene foam gasket; (4, 5, 6) receiver tube, delivery tube, and tent-shaped paper evaporator, which comprise the preconditioning system (for adding known small amounts of water and other volatiles); (7) motor-driven fan to hasten evaporation; (8) combined support for evaporator and positioner for the bottom clamps of the chromatograms; (9) porcelain solvent trough (adjustable in height to permit variation in length of feeder wick).

conditions to be described in Part 4 of this series. While there is probably no full substitute for a molded glass box as regards resistance to attack by any conceivable chromatographic solvent, the laminated box of the type 4 unit offers some advantages over a glass jar; for example, the walls provide better thermal insulation and hence protection from temporary disturbances in temperature, e.g. during opening of the constant temperature cabinet. Furthermore, an effective vapor-tight closure was evolved for the type 4 unit (which is not easily achieved with a large glass box).

The top closure of the type 4 unit consists of a sheet of plate glass which rests upon a flat gasket, cut in one piece from a 3/16-in. sheet of soft neoprene foam,

the neoprene forming the continuous phase of the foam. This is sealed with neoprene cement to the flat top of the walls. The glass lid is provided with a hole 3/8 in. in diameter, through which the solvent is added, this being closed normally by a glass coverslip backed with adhesive tape. A heavy insulating cover (3/4-in. plywood, faced with cork linoleum and backed with 1/8-in. steel plate) rests on top of the glass lid.

(*b*) *The Solvent Trough*

It was found to be important to be able to regulate accurately the rate of supply of solvent to the chromatogram. An excessive rate of irrigation is known to lead often to large and elongated spots, particularly of faster-running solutes and to impaired resolutions, as has been recognized by other workers (e.g. 21, 25). Defects from this cause are seen frequently on chromatograms hung directly over the edge of the usual shallow form of solvent trough, especially when the latter is filled to capacity with solvent, as for a long irrigation, and the imperfections are accentuated with thick, porous papers, and with solvents of low viscosity.

Effective control of the irrigation rate was obtained by modification of the shape of the solvent trough and the manner of mounting the chromatogram in it. One suitable design of trough is shown in Figs. 2 and 4 and another in Fig. 3. The trough is relatively deep and has a large flat bottom. The chromatograms are mounted so that the solvent travels first up a vertical limb of the paper, then horizontally and finally downwards before it reaches the 'starting line' along which the solutes have been 'spotted'. The resistance to solvent flow presented by this length of paper (ca. 10 cm) ensures that the flow will have diminished appreciably by the time the solvent reaches the starting line.

For some systems, further restriction in the rate of flow is advantageous and this is achieved reproducibly by providing a restricting 'feeder wick', a device which in one form or another has been advocated by various authors (15, 21, 25, 26). In the present work, a wick of thinner paper is stitched to the actual chromatogram by sewing machine, and this wick forms the vertical limb up which the solvent travels from the trough. Figure 4 shows a chromatogram with attached wick, suspended by three glass rods secured along the line of stitching. It is important that the rings holding these rods should be loose, to avoid restriction of solvent flow by squeezing of the paper at the two sides; narrow rings cut from polyethylene tubing or bent from thin strips of stainless steel are used for the purpose. The bottom edge of the wick should be kept from approaching the wall of the trough and so shortening the effective path of ascent of solvent. Provision is made in both designs of trough for varying the length of the wick either by raising the mounting rods of the chromatograms on suitable spacers such as that shown in Fig. 4, or by raising or lowering the height of the trough by a simple adjustment. In practice, three lengths of feeder wick (measured from the line of stitching to the bottom of the trough)

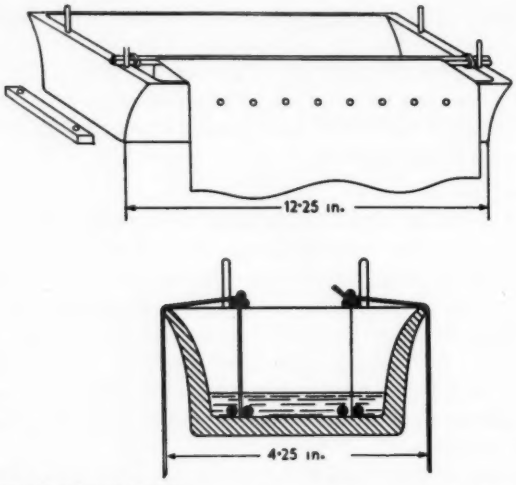

FIG. 4. Porcelain solvent trough.
Upper perspective drawing shows single chromatogram in position, being held between three glass rods which rest against pins. Spacer bars, as shown at left, can be slipped over the pins to raise the support rods (to vary wick lengths).
Lower section shows two chromatograms in position. Chromatogram on the right has stitched-on 'feeder wick' of this paper to restrict solvent flow; that on the left has wick formed by end of chromatogram itself.

have been used, namely, 48, 54, and 60 mm.

By selection of the length of wick and type of paper used, wide differences in the rate of solvent flow are obtained and a rate suitable for a given system is readily determined. Finer adjustments of flow rate, e.g. to produce desired separations in operationally convenient periods (e.g. 16 or 40 hours) may be made by smaller changes in the effective length of wick, which can be brought about by altering the volume of solvent added to the trough. For example, in the case of the trough shown in Fig. 4, the effective wick lengths with different volumes of added solvent are shown in Table I.

TABLE I

Effective length of feeder wick with different volumes of solvent (applicable to trough illustrated in Fig. 4)

Total length* of wick (mm)	Effective length of wick (mm)† with additions of solvent as follows: Vol. (ml)					
	325	300	275	250	225	200
48	31	32	33.5	34.5	36	37
54	37	38	39.5	40.5	42	45
60				46.5	48	49

*Measured from line of stitching to bottom of trough.
†Measured from line of stitching to surface of solvent.

(c) Preconditioning of the System

It is recognized generally that the composition of the vapor phase within the chromatographic unit may exert a profound influence upon the operation of a given system and a range of effects resulting from interactions between the vapor phase and the partitioning phases on the paper have been reported (2, 4, 15, 18, 20, 22). The need for some form of preconditioning before addition of the chromatographic solvent is accepted widely and the commonest practice has been to provide a pool of the solvent which is to be used for the irrigation or, in the case of two-phase mixtures, either the water-rich phase or a mixture of both phases. This conditioning liquid is usually exposed in a tray or in the bottom of the actual chromatographic container, this surface being supplemented sometimes with extra evaporators of cloth or paper, and sometimes with mechanical means for causing air movement inside the closed unit. Depending upon the efficiency of such arrangements, preconditioning periods ranging from 1 to 48 hours have been used by different workers. With some solvent systems, and under particular climatic conditions, the need for preconditioning may be less important (e.g. 4).

The present investigation was begun with a tacit assumption (proved later to be erroneous) that full pre-equilibration of the vapor phase and filter paper with the chromatographic solvent would represent ideal practice, on the grounds that this would minimize changes in composition of the liquid and gel partitioning phases on the chromatogram during the irrigation (p. 122). The conditioning equipment shown in the type 3 unit (Fig. 2) represented an efficient but cumbersome arrangement for equilibrating the closed systems with the vapors from any chosen conditioning liquid. An upper reservoir of about 1-liter capacity was kept filled with the conditioning liquid and this flowed continuously down two curtains of Whatman No. 3 paper, the drip being collected in a tray below. A small electrically driven fan caused air to circulate over the wet curtains for the duration of the conditioning period. The need for the continuous fresh supply of liquid to the curtains had become apparent in experiments with earlier forms of equipment when it was found that pools of solvent left within units underwent drastic changes in composition, e.g. a rapid decrease in water content of alcohol–water solutions due to anomalous vapor pressure relations (28); this caused progressive changes in the patterns of separation if successive pairs of chromatograms were run in the same unit without replacement of the solvent pool each time, an expedient which carried disadvantages.

The efficient equilibrating mechanism of the type 3 unit facilitated a study of the influence of varying the initial composition of the vapor phase upon different chromatographic systems, with adequate control of other factors at the same time. A number of the results, although consistent with some earlier experience and with certain isolated observations in the literature, were unexpected, and in the end they led to the complete abandonment of the conventional method of preconditioning of chromatographic systems as

embodied in the type 3 and earlier units. Contrary to the tacit assumption mentioned above, it was found that full pre-equilibration of the system with the volatile constituents of the irrigating solvent was not necessarily an optimum initial state and that the complete omission of a particular solvent constituent might induce desirable features in a pattern, e.g. the omission of ethanol from the vapor phase when a solvent containing propanol, ethanol, and aqueous buffer was to be used. It became evident, in fact, that controlled evaporation of selected solvent constituents during the irrigation with the induction of a gradient of conditions on the chromatogram might be used to bring about beneficial changes in the composition of the partitioning phases during the irrigation process.

A further important consideration, of which the full significance became clear only after the work with the type 3 unit was completed, relates to the initial water content of the system. There were various indications supporting the view of McFarren (20) that addition of water is the major operative result of the usual preconditioning treatments. It became clear, moreover, that it is the water imbibed in the paper at commencement of the irrigation process which is a most important determinant of the operation of the chromatogram. The water-holding capacity of the air enclosed within the unit (57 liters in type 3 and 4 units) is relatively small, being only 2.1 g at full saturation at the operating temperature, while the water-holding capacity of a pair of chromatograms is several times this amount. It became clear that widely varying amounts of imbibed water ranging from about 3 g under dry winter conditions to as much as 12 g during humid summer weather are introduced into the system with the chromatograms and this provided an explanation for the frequently observed deterioration during humid weather of apparently well-stabilized chromatographic patterns. When relatively moist papers are introduced into a unit, the effect of the usual preconditioning treatments may be to produce an excessive initial water content resulting in serious and characteristic chromatographic defects.

Observations extending over several years pointed to the need for a more positive method of standardizing the initial state of a chromatographic system which would take into account the varying amounts of water present in the filter papers. It was clearly desirable that the total initial content of all volatile constituents of the system, including the water imbibed in the cellulose, should be adjusted to some predetermined state judged to be optimum for the particular system. Two approaches to the problem were developed and compared.

The first approach, which deserves only brief mention, consisted of passing a stream of conditioned air through the unit, this being bubbled first through an appropriate solution to control its content of moisture and other vapors. Although effective under fairly uniform conditions of ambient humidity, no simple means was found whereby this method could be used to compensate for large variations in the amounts of imbibed water introduced with the chroma-

tograms.

The second approach, which led to the procedure finally adopted, and to the form of conditioning equipment provided in the type 4 unit (cf. Fig. 3), is simple and versatile. This is based upon the direct addition of the amounts of water and of any other volatile constituents which are required to bring the system to a specified initial state. In the case of water, this involves making an approximate estimate, by one of several rapid methods, of the amount of imbibed water present in the two chromatographic papers placed in the unit; then additional water is added to make up a specified total amount. The volumes of water and other liquids required are added by pipette into the external receiver tube provided in the type 4 unit from which, as may be seen in Fig. 3, the liquid is discharged on to a small paper evaporator which is supported near the bottom of the unit before the motor-driven fan. The total volume of the added liquid, falling usually in the range 5–15 ml, is distributed rapidly by operating the motor-driven fan for a specified short period, normally 30 minutes.

The great advantages stemming from this new approach to the preconditioning of chromatographic systems have been its versatility and operational simplicity and a great improvement in the reproducibility of chromatographic patterns irrespective of the widest variations in atmospheric humidity. A requirement of the method is a knowledge of the moisture content of the chromatographic papers when placed in the unit. Two rapid approximate methods for this estimation will be described in Part 4 of this series, as well as other details in the practical use of the method. The new approach provides for the easy exploration of the influence of variations in the initial content of different volatiles upon any particular chromatographic system and for an unambiguous specification of the optimum initial state when this has been established.

Preparation of the Chromatogram

Grade and Purity of the Paper

Five batches of Whatman No. 3 paper (mold made, not No. 3 MM), purchased between 1951 and 1959, have formed the basis of the chromatography described in Parts 1–8 of this series of communications.* In the past this paper has been purchased in sheets 29½ in. × 22 in., which have been cut lengthwise by guillotine to give the final sheets of 29½ × 11 in.; the latter size can now be obtained from the supplier (Messrs. H. Reeve Angel and Co., Ltd.).

Grade No. 3 was selected originally in 1948 as tolerating heavier loadings of

*(Note added Sept. 29, 1960.) An extensive series of experiments carried out in collaboration with Messrs. W. and R. Balston Ltd., Maidstone, Kent, and Messrs. H. Reeve Angel and Co., London and Clifton, New Jersey, has now led to a modified Grade No. 3 filter paper which, in addition to exhibiting low ninhydrin 'blank' values, has a special texture which is particularly suited for use in the new 'high resolution' chromatographic systems to be described. This recommended grade of paper, which is referred to as Whatman No. 3 HR, is now available for this work in sheets 29½ in. × 11 in., cut with the long dimension parallel to the machine direction.

test substances and yielding more compact spots than a range of other papers then tested. It was found desirable in various applications to purify the paper before use, and a number of procedures culminating in the rigorous washing process described in Part 1 (1) were developed. So far as amino compounds are concerned, the advantages of the washed paper were (*a*) a marked improvement in the chromatographic separation of amino acids and peptides in the simple unbuffered systems then in use, and (*b*) a large reduction in the 'non-ammonia ninhydrin values' given by the paper. It was found subsequently when the improved buffered systems were developed that the chromatographic advantages of the washing treatment were less marked and ultimately insignificant. However, in the interests of analytical accuracy, washing of the paper was necessary for quantitative work until 1957, when a marked improvement was observed in the purity of the paper as purchased.

A summary of some analytical observations on the different batches of paper is given in Table II. The paper of the earlier batches (A, B, and C) gave high and variable ninhydrin values, whereas the last two batches (D and E)

TABLE II

Purity of Whatman No. 3 paper

Batch	Year of purchase	Non-ammonia ninhydrin values (×100)* Mean	Non-ammonia ninhydrin values (×100)* Range	Ultraviolet absorbancy values (×100)† Mean	Ultraviolet absorbancy values (×100)† Range
(Analyzed as purchased)					
A	(1951)	Ca. 7	4.0–12.0	Ca. 6.0	4.0–7.0
B	(1953)				
C	(1955)				
D	(1957)	2.7	1.1–4.0	3.9	2.2–5.4
E	(1959)	2.3	1.4–4.5	3.6	2.4–6.2
(Analyzed after purification)‡					
A and B		Ca. 3		—	—
C		2.3	1.0–4.1	4.2	2.8–5.8
D		1.3	0.2–2.4	5.0	3.8–7.0

*Absorbancy at 570 mμ determined with ninhydrin–hydrindantin reagent under conditions to be described in Part 5 of this series, using 0.2 g paper and final volume 10 ml.
†Absorbancy at 260 mμ determined on 0.26 g paper in 10 ml water, extracted 30 minutes with gentle rocking.
‡Purified by prolonged washing as described earlier (1).

gave mean values only one-third as great and showed smaller variability; the range of variation in the earlier and the later batches would correspond to about 0.04 and 0.015 μmole amino acid, respectively, in the 'paper blank' value for a medium-sized chromatographic spot. The purity of the later batches was considerably improved as judged by the ultraviolet absorbance values also.

The effect of the rigorous washing treatment applied to the early batches (A, B, and C) was greatly to reduce the ninhydrin values and the range of variation; in the case of batch D, which showed high purity as purchased, washing produced a further decrease in the ninhydrin values (but inexplicably a small increase in the ultraviolet absorbance values). Batches D and E as

purchased showed approximately the same level of purity as had been obtained by the purification in the laboratory of the earlier batches.

With the increased purity of the paper during the past 2 years, it has become possible to dispense with the washing treatment previously used without loss in either chromatographic resolution or analytical accuracy. The only purification to which the paper is subjected at present forms an integral and convenient initial step in the process whereby it is impregnated with specified solutions of buffers and other salts.

The Buffering of Chromatographic Papers

In the pretreatment with buffer solutions, it is desirable to achieve a uniform distribution of the prescribed salt mixture over the whole area of each paper. To this end it is essential, after wetting the papers with a specified solution, to remove all free-running solution before hanging them up to dry. This is effected by brief suction on a perforated suction platform, akin to a rectangular Buchner filter without walls as illustrated in Fig. 5. While this operation can be carried

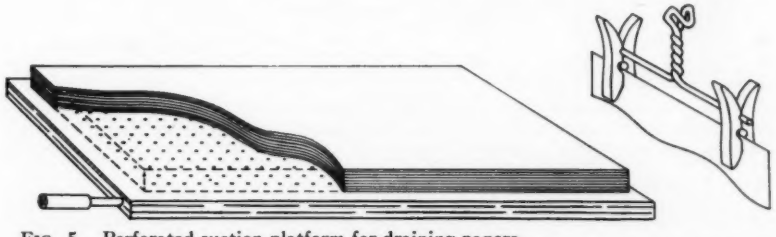

FIG. 5. Perforated suction platform for draining papers.
This is used for alternately soaking papers in buffer solutions and draining off all excess liquid before hanging papers to dry. Hangers constructed as shown on the right facilitate handling of the wet papers.

out on a few sheets (hand-dipped and then spread individually for drainage by suction) a more satisfactory product is obtained by a procedure developed for treating batches of about 35 sheets.

A pile of 35 dry sheets of the No. 3 HR paper, neatly stacked, is clamped at one end to a sheet of perspex (32½ in. × 12 in. × ¼ in. thick) by means of a wooden yoke with foam rubber grips. At this clamped end, each paper is inscribed with a distinguishing pencil mark. (In the subsequent process, this end of the paper will become contaminated to some extent with impurities washed out of the remainder of the sheet; hence this marked end ultimately forms the bottom of a chromatogram to be folded into the absorption pad where the presence of impurities is of no consequence.)

The perspex sheet with attached papers is now placed on a stand as shown in Fig. 6 with the papers hanging freely into a tall rectangular tank (12¼ in. × 3¼ in. by 15 in. high, outside dimensions) constructed of ¼-inch perspex. The buffer solution (4 liters) is run gently into the bottom of the tank through a long-stemmed funnel and the solution is permitted to ascend the papers by

FIGURE 6

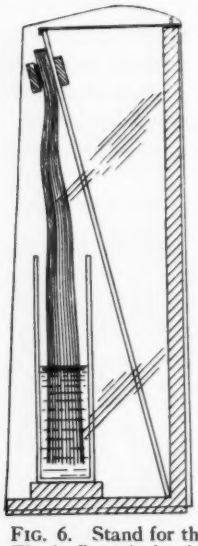

FIGURE 7 Ttemplates

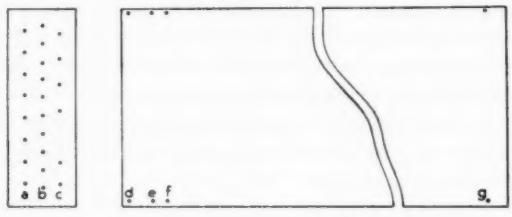

FIGURE 8

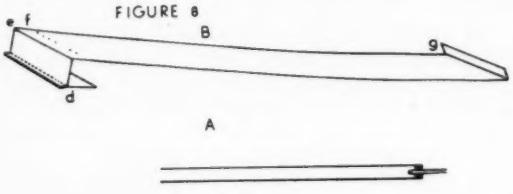

FIG. 6. Stand for the wetting of batches of paper.

The buffer solution is allowed to ascend the papers by capillarity so that wrinkles are stretched out, permitting a compact block to be formed on the suction platform shown in Fig. 5.

FIG. 7. Templates.

Accuracy in laying-out chromatograms facilitates proper fit into mountings. Convenient templates of $\frac{1}{8}$-in. perspex sheet are shown. That on the right, 73×27 cm, is the full size of the chromatogram and is used to mark the positions of lines at *d*, *e*, *f*, and *g* by pencilled dots through the drilled holes indicated, these being, respectively, *d*, line for stitching on the feeder wick; *e*, line of folding (to correspond to lip of trough in Fig. 4); *f*, line for spotting of solutes; *g*, line of folding to insert into absorption pad (cf. Fig. 9). Template at left is drilled for rapid marking out of convenient spacings of spots along the line *f*. For example, row *a* contains 8 spots spaced at 30 mm, row *b*, 10 spots spaced at 24 mm, and row *c*, a special arrangement of 6 spots useful for an analytical procedure to be described in Part 4 of this series. An additional template for uniform marking of feeder wicks may be found desirable.

FIG. 8. A. Capillary spotting pipette, self-filling and self-emptying. B. Chromatogram in position for spotting. Feeder wick is attached (by stitching along line *d*) and folds have been made at *e* and *g*. Spotting line *f* is held away from bench.

capillarity for 16–20 hours, the whole apparatus being covered snugly in a plastic cover to prevent both evaporation and contamination by dust. This process achieves two purposes in the most convenient method so far devised. The first is to wet the sheets without wrinkling, the increase in length being 'stretched out' of the free-hanging papers by their own weight; freedom from wrinkles is essential in order subsequently to form a tightly packed pile on the suction platform without damage to the paper structure. The second advantage of this procedure is that a significant amount of impurity is washed up to the top end during the capillary ascent of the buffer solution. When the papers are wetted to the top, the perspex sheet with the attached papers is lifted slowly

out of the tank and laid flat on the bench. The yoke is then removed and the pile of wet papers is transferred to the suction platform by first placing the latter on top and inverting with the wet papers supported between suction platform and perspex sheet. The paper is now covered with a sheet of polyethylene and, with gentle suction applied by filter pump, it is pressed down gently by hand until, as observed by a manometer, the holes are all sealed. The suction is now discontinued and the pile of papers is full wetted by pouring on slowly 350 ml of the buffer. After 5 minutes, excess solution is drawn off by applying the suction for about one minute, the platform being tilted suitably for drainage. After this first full wetting, the operation of adding 350 ml of fresh buffer, soaking for 5 minutes, and then draining off free solution under suction is repeated three times; the whole 350 ml added should be recovered each time to ensure uniform impregnation and washing of the whole pile.

The papers, devoid of free-running solution, are now lifted individually from the pile by attaching hangers of the type illustrated in Fig. 5, and are hung to dry in a clean space, preferably free from draughts. When dry they are packed in protective polyethylene envelopes and pressed flat by weighting.

The Chromatogram

The assembled chromatogram, as mounted in position in the unit, has been illustrated already in Figs. 2, 3, and 4. It consists of three paper parts, namely: the main chromatographic paper; the feeder wick, often of Whatman No. 1 paper, which is attached by stitching across the top edge of the chromatogram; and the absorption pad, which is held by a tubular clamp across the bottom edge (cf. Fig. 9C). The chromatogram is suspended from the trough by means of three glass rods; the tubular clamp serves as a positioner at the bottom and also as a small weight (ca. 100 g) to hold the chromatogram taut (cf. Figs. 2, 3, and 4).

It is important that the chromatogram should fit properly into the unit and this is facilitated by preparing a permanent template of $\frac{1}{8}$-in. perspex sheet, which is used to mark out each chromatogram. This is used to outline if necessary the final trimmed size of the sheet (11 in. $\times$ 29$\frac{1}{2}$ in.) and to mark with a soft pencil through drilled holes in the template the positions of the line for stitching on the feeder wick, the line of folding where the chromatogram will hang over the lip of the trough or the glass supporting rods (cf. Figs. 2–4), the 'starting line' along which the solutes are to be spotted, and finally a fold near the bottom to form a flap to be inserted inside the absorption pad. It is convenient to prepare a subsidiary template for marking off, along the starting line, various spacings for the spotting points which may be found convenient for different purposes (cf. Fig. 7). A simple template for marking out the feeder wicks of different lengths is also useful.

For spotting on the solutes, a simple capillary pipette, made by drawing out glass tubing into the general form shown in Fig. 8A is used. These are made to deliver volumes ranging from 1 to 8 μl; they are filled by capillarity and emptied

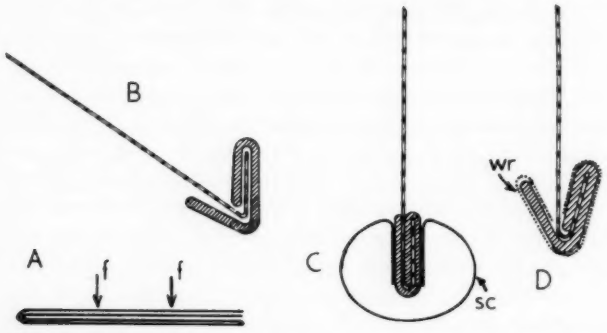

FIG. 9. The absorption pad.
A. Half-sheet of No. 3 paper (14½ in. × 11 in.) is made into 4-ply pad of about 3¾ in. × 11 in. This is then folded into three along *f*, *f*.
B. Folded end of chromatogram is inserted into middle of pad to ensure capillary continuity.
C. Pad is clamped by light tubular clamp (*sc*) about 9 in. long, which is shown in section only.
D. In latest procedure for preconditioning, the pad is wrapped snugly in polyethylene sheet (*wr*) (15 in. × 6 in.), the ends being folded over and secured by the pressure of the tubular clamp seen in C.

similarly on touching the filter paper. Pipettes with volumes of about 4 μl, which are most commonly used, are accurate to approximately 0.5% when used carefully. Calibration is done by several weighings of the capillary pipette (without its holder) filled with water and then after it is emptied by touching filter paper.

For the operation of spotting, it is convenient to fold the chromatogram with wick attached as shown in Fig. 8B and place it on a clean bench; the spotting is done with the starting line nearest the operator. It is important in quantitative work that the paper should not be pressed down to touch the bench surface at the point of spotting since this may cause serious loss of the solution by its wetting through the chromatogram.

The final step, before the chromatogram is placed in the unit, is to attach an absorption pad to the bottom. This is a commonly used device to provide continuing suction capacity additional to that of the chromatogram itself; otherwise the flow rate falls markedly when the solvent front reaches the bottom of the chromatogram. For most of the work, an absorption pad made from one-half of the standard sheet of No. 3 Grade paper (i.e. 14½ in. × 11 in.) has been used. It is essential that the chromatogram should be in close contact with the absorption pad across its whole width to ensure uniform suction. An effective method of ensuring this capillary continuity is shown in Fig. 9, A to D. Figure 9C shows in cross section the tubular clamp of light stainless steel which is slipped over the folded pad to hold it in position; this clamp is 9¼ in. long. Figure 8D shows an additional feature used in conjunction with the final method of preconditioning of the system to be described in Part 4, namely a poly-

ethylene wrapper which surrounds the absorption pad snugly and which is held in place by the clamp. This forms a sufficiently effective barrier to water vapor to permit the initial moisture content of the paper comprising the absorption pad to be ignored in specifying and adjusting the initial water content of the system. This resolved troublesome complications arising from the slow equilibration of the tightly folded pads.

Details of the procedures and conditions for the preconditioning and irrigation of chromatograms in the types 3 and 4 units are to be described in Parts 3 and 4 of this series. The further point deserves mention that the removal of the wet chromatograms at the end of the irrigation is done conveniently by first attaching one of the hangers shown in Fig. 5, the two pegs being clipped over the mounting rods, the whole is then lifted out of the unit and is suspended on a rack under the vapor hood to dry at room temperature (cf. previous section). The absorption pads are detached from the chromatograms immediately to prevent backflow of solvent during the drying. The pads are unfolded for drying, and are used repeatedly.

Discussion

This communication is intended to provide a background for presenting, in subsequent parts, the results of rather extensive investigations into the separation of amino acids and, later, nucleotides on the paper chromatogram. These investigations, undertaken with the purely practical object of sharpening an analytical tool important in connection with certain biochemical problems under study in this laboratory, have far outgrown all original intentions and have led to developments at various levels which promise to be more generally useful. Two of these developments, very different in character, but having in common the fact that they may contribute in a general way to the improvement of paper chromatography, have been presented in this communication. The first of these is the emergence of a more detailed working hypothesis regarding the physical organization and functioning of the paper chromatographic system. The second, at the practical level, is the progress which has been made toward the design of chromatographic equipment permitting closer control of a number of factors which influence the operation of the system. The relevance of these developments to the possibilities of securing improved resolutions of particular groups of compounds in different paper chromatographic systems is to be illustrated in the subsequent communications.

Acknowledgments

The chromatographic studies described in this communication have been carried out during the past six years in connection with a number of projects supported by the National Research Council of Canada, the National Cancer Institute of Canada, and, in the early stages, the J. P. Bickell Foundation; it is a pleasure to acknowledge the support given by these bodies.

The writer wishes to thank a number of colleagues who have helped at

different times and in different ways in the evolution of the equipment which has been described, especially Dr. F. A. Isherwood, Dr. A. T. Matheson, Miss E. H. M. Wade, Dr. C. K. Harris, and Dr. M. A. Moscarello, and finally to thank Professor C. Barnes of the Physics Department, University of Toronto, for a most helpful discussion of physical aspects of the problem.

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QUANTITATIVE CHROMATOGRAPHIC METHODS

PART 3. FACTORS CONTROLLING THE PATTERNS OF SEPARATION OF THE AMINO ACIDS ON PAPER CHROMATOGRAMS[1]

E. H. M. WADE,[2] A. T. MATHESON,[3] AND C. S. HANES

Abstract

The possibility of developing a complementary pair or trio of one-dimensional chromatographic systems capable of separating reliably all the common amino acids is considered. In order to secure the required substantial improvements in both the resolving power and the reproducibility which would be essential, the approach is adopted of attempting with selected systems to exploit as fully as possible the feature of adjustability in the patterns of separation of the amino acids. As a class it seems that systems consisting of buffered miscible solvents used with papers pretreated with the same buffers are especially promising in this respect. A representative family of systems of this class (based on *n*-propanol, ethanol, and aqueous pyrophosphate buffers containing sodium chloride) is selected for the systematic study of the influence of a number of variables upon the separations. As a result great improvements in resolution became possible, and the main objectives of the investigation were shown to be within reach.

Introduction

The object of this investigation, as was indicated in the preceding communication (1), was to survey the possibility of securing substantial improvements in the separation of amino acids on one-dimensional paper chromatograms to serve as a basis for a dependable system of quantitative analysis.

Since the classical work of Consden, Gordon, and Martin (2), two-dimensional paper chromatography has been used widely to separate and identify the amino acids present in complex mixtures, and a number of 'contrasting pairs' of solvents have been suggested for the purpose, e.g. phenol–water followed by collidine–lutidine–water (3, 4), phenol–water followed by *n*-propanol–water (5), *n*-butanol–acetic–water followed by phenol – cresol – aq. borate at pH 9.3 (6), phenol – aq. HCl – KCl at pH 1.0 followed by *n*-butanol–acetic–water (7). The sole advantage of the two-dimensional procedure lies in the enhanced resolving power which is obtained. For example, the above pairs of solvents separate all or most of the 20 common amino acids on two-dimensional chromatograms, whereas under the same conditions no one of these solvents when used on one-dimensional chromatograms yields more than six to eight spots. Despite its magnification of resolving power, two-dimensional chromatography carries inherent disadvantages for quantitative work. The final chromatogram shows unavoidably the combined defects of both the solvents used to produce it. Since in all available solvents some amino acids tend to show trailing or other chromatographic defects, there has been little hope of

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separating all the amino acids in the form of the compact circular spot which represents the ideal for quantitative analysis. In addition, the use of a particular solvent required for certain separations may introduce special analytical difficulties which are likely, on the two-dimensional chromatogram, to affect the determinations of all the amino acids. For example, as will be discussed later, amino acids separated in phenolic solvents tend to give low yields of Ruheman's purple in the quantitative ninhydrin–hydrindantin procedures; yet a phenolic solvent is included usually in the solvent pair used in two-dimensional chromatography, owing to its capacity to separate serine and glycine. Finally, there is the serious limitation that only a single small specimen can be separated on a two-dimensional chromatogram.

It is evident that one-dimensional chromatography offers important advantages if adequate separations of the components can be obtained. Especially important from an analytical point of view is the fact that a number of specimens, including different loadings of each mixture to be analyzed, can be applied side by side; spots of each amino acid can then be selected for analysis within the optimum range of the analytical method and recoveries from different loadings are readily compared. Moreover, the possibility of chromatographing relatively large quantities of material, applied in a band across the one-dimensional chromatogram, becomes a feature of obvious importance whenever unidentified compounds are encountered in the mixtures to be analyzed.

Patterns of Separation of the Amino Acids on One-Dimensional Chromatograms

Probably no group of compounds has been studied more extensively than the amino acids as regards chromatographic behavior. Accordingly, a wealth of information was available at the outset regarding the patterns of separation of the amino acids obtainable with a great variety of one-dimensional systems. This served to define the objectives of the present study and to suggest general lines of approach. For example other workers, recognizing the potential advantages of the one-dimensional chromatogram, especially for quantitative work, had sought similarly to develop complementary sets of one-dimensional systems for separating all the amino acids (8, 9, 10, 11). McFarren (8), after testing some 60 combinations of partially water-miscible solvents with various aqueous buffers ranging from pH 1 to 12, described a set of seven systems capable of separating cleanly all the common amino acids. Redfield and Barron (9) proposed another set of five systems. Neither of these procedures found extensive application, however, owing doubtless to their complexity, and it seemed that, for practicability, any proposed analytical procedure should require no more than two, or at most three, different systems. Progress towards this objective had already been made. Fowden (10) proposed a set of three systems which resolved most of the amino acids, but which required 5 days for the irrigation. Roland and Gross (11), having developed a one-dimensional system of superior resolving power (to be described below), were able to

propose a set of two systems requiring 40–48 hours for the irrigation which resolved 16 amino acids and they proposed additional systems for separating other amino acids. Both these procedures showed some limitations; for example, both employed a phenolic system for resolving six amino acids and the quantitative determinations of these would be rendered somewhat unreliable, and both failed to separate some few amino acids of importance. Moreover, the superior system of Roland and Gross, while indicating strikingly the potentialities for increased resolving power of the paper chromatogram, was said to show variability which required adjustment of solvent composition and irrigation time, and it suffered from the disadvantage of using an ammoniacal solvent undesirable in a laboratory in which determinations are to be made by means of a ninhydrin–hydrindantin reagent.

It seemed that any closer approach to the ideal pair or trio of systems would require great improvements in both resolving power and reproducibility of the one-dimensional chromatogram. Consideration of general experience and of the specific patterns of separation recorded in the literature suggested that such improvements would come, if at all, not from the discovery of some new solvent system of spectacular superiority, but more probably from the progressive adjustment and refinement of a few selected systems on the basis of closer knowledge and control of different factors influencing their operation.

In Fig. 1 is shown diagrammatically a selection of the patterns of separation of amino acids reported for different systems.* The resolutions indicated for these range from 7 to 15 separated amino acids or groups of amino acids; patterns E and G represent the most refined separations so far encountered in the literature. The remarkable resolution shown in pattern G was achieved by the special technique, which has been used successfully by different workers (12, 13, 14, 15), of repeated irrigation of the chromatogram with the same solvent, *n*-butanol – acetic acid – water, in this case, the solvent being dried off after each irrigation. By comparison with pattern F, it will be seen that this procedure, although lengthy, is capable of enhancing greatly the final resolving power of a particular system. A commoner device for securing improved resolution of the slower-moving amino acids has been merely to prolong the period of irrigation, sometimes allowing fast-running compounds to run off the paper (see, for example, refs. 9, 16).

The patterns A to G shown in Fig. 1, and others not illustrated, appear superficially to present great diversity, but closer study reveals underlying features of similarity. The 'neutral' aliphatic amino acids, for example, exhibit an almost constant order in all the solvents, this order (from slow to fast) being serine + glycine, threonine, alanine, valine, methionine, isoleucine, and leucine. The only exceptions appear to be some variability in the positioning of valine and methionine and the fact that the collidine–lutidine–water solvent

*The following abbreviations for the amino acids are used in Figs. 1–8: AA, aspartic acid; AL, alanine; AR, arginine; CA, cysteic acid; CC, cystine; G, glycine; GA, glutamic acid; HI, histidine; HP, hydroxyproline; IL, isoleucine; L, leucine; LY, lysine; M, methionine; PA, phenylalanine; PR, proline; S, serine; TH, threonine; TR, tryptophan; TY, tyrosine; V, valine.

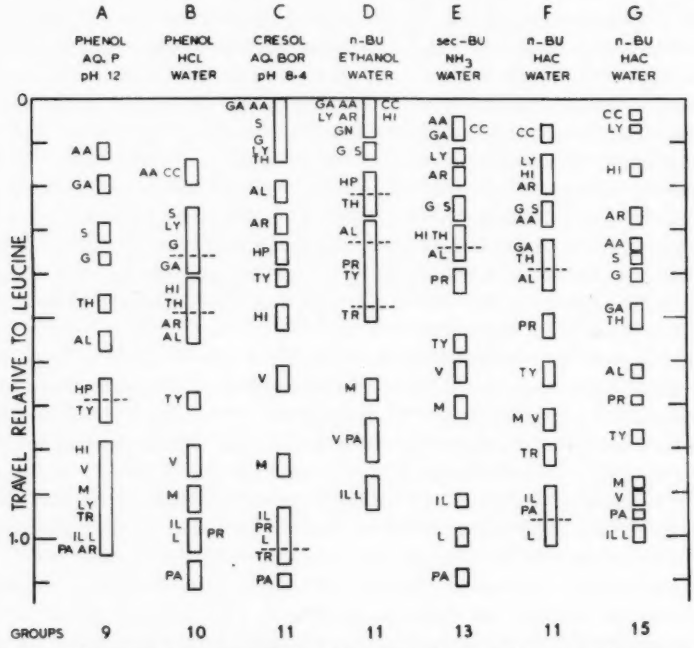

FIG. 1. Diagrammatic representation of patterns of separation of published systems.
A. Phenol – aq. phosphate buffer at pH 12 (8).
B. Phenol – hydrochloric acid – water (7).
C. Phenol – aq. borate buffer at pH 8.4 (8).
D. *n*-Butanol–ethanol–water (12, 13).
E. *sec*-Butanol–ammonia–water (11).
F. *n*-Butanol – acetic acid – water (6).
G. *n*-Butanol–acetic–water (14, 15).
Dotted lines represent recognizable divisions between contiguous spots. The number of groups separated, indicated at the bottom of each pattern, includes these imperfectly separated groups.

often used in two-dimensional systems (17) is unique in giving the order glycine, serine, alanine, threonine, i.e. relative retardation of the two hydroxylated amino acids. The neutral amino acids with cyclic R-groups show more variation in their order of travel and in their distribution relative to the other amino acids, since they are differentially accelerated in solvents of aromatic character. The greatest variability, however, is shown by the 'acidic' amino acids (aspartic and glutamic acids), and the basic amino acids (lysine, histidine, arginine, and ornithine). This is long recognized as being due to retardation of the basic amino acids and acceleration of the acidic ones in acid solvents and to the reverse effects in alkaline solvents (2). These effects, reflecting changes in hydrophilic character associated with ionization, apply with variations to widely different solvents and are illustrated by patterns A and B and also by

E and F in Fig. 1. This is the best known example of the manner in which the pattern of separation may be adjusted by the conscious variation of a particular factor. There are numerous indications in the literature that the patterns of separation may be altered and adjusted in characteristic ways by controlled variations in other factors, including proportions of components of the solvent mixture, presence of buffers and salts, properties of the paper, and nature of the atmosphere within the chromatographic unit. In fact, it seemed that the best hope of securing the required substantial improvement of the one-dimensional chromatogram might lie in attempting to exploit this feature of adjustability of selected systems by first studying systematically the influence of all recognizable factors upon the patterns of separation and then controlling each factor in turn to secure desirable characteristics.

Equipment and Methods

Equipment

Some general procedures and new forms of equipment used in this and subsequent studies have been described in Part 2 of this series (1).

Brief reference will be made in general terms to a preliminary survey carried out with earlier forms of equipment but the main results to be presented were obtained by the use of the type 3 unit. This included the final type of solvent trough and fittings to accommodate the standard size of chromatogram. The type 3 unit was provided with the rather cumbersome but efficient conditioning system described in the preceding paper whereby the atmosphere within the unit could be brought rapidly into equilibrium with any selected 'conditioning liquid'.

Conditioning of the System

In first setting up a chromatographic unit for use with a particular alcoholic conditioning liquid, an initial conditioning period of 24 hours was allowed; thereafter the conditioning liquid was refreshed only 4 hours before the unit was to be used. The conditioning liquid used in each system is specified (cf. Table I). After the pair of chromatograms were hung in place and the unit closed, the motor was run for 30 minutes before the irrigating solvent was added.

In the case of the phenolic systems a simpler procedure was adequate. The conditioning equipment of the type 3 unit was removed and replaced by a shallow tray which was kept filled with water on the floor of the unit. Again a 30-minute period of fanning was allowed after the chromatograms were suspended.

Preparation of the Chromatograms

The separations to be illustrated were obtained in all cases by the use of Whatman No. 3 paper, without purification but pretreated by impregnation with aqueous buffer solutions which are to be specified for each system (cf. Tables I and II). Excess buffer solution was removed from the papers by suction in the manner described in Part 2 (1) before they were hung to dry.

Each chromatogram (11 in. × 29½ in.) was provided with a 'feeder wick' of No. 1 Whatman paper (11 in. × 3 in.) stitched across the top and with an absorption pad consisting of a folded half-sheet of No. 3 Whatman (11 in. × 14¼ in.) fixed to the bottom as described earlier (1).

Appropriate known mixtures of amino acids for identifications and for the study of effects of concentrations, volumes applied, presence of salts, etc., were applied along the 'starting line'. The papers were suspended, always in pairs, in the type 3 unit.

Preparation of Solvents

Sodium chloride sufficient to make up the specified amount (cf. Table I), was dissolved in the aqueous components of the solvent prior to addition of the non-aqueous components. This caused precipitation of excess sodium pyrophosphate or other salts. Accordingly the mixture was normally allowed to stand overnight at 34° C (inside the constant temperature cabinet) and only the clear supernatant was used as the irrigating solvent.

Results

Preliminary Survey of Buffered Solvents

The advantage of including buffers in chromatographic systems for separating amino acids had been indicated clearly by the observations of various workers (18, 19, 20, 21) but especially by those of McFarren (8). In these cases the use of buffers had been tested in two-phase solvent systems (i.e. formed with partially miscible solvents) despite the fact that a number of fully miscible solvents unbuffered were being used with comparative success (5, 9, 22, 23, 24, 25). In view of advantages experienced in using miscible solvents, particularly the greater ease of adjustment of composition and properties, and less tendency for the separation of phases on the paper, an extensive exploration was undertaken of combinations of miscible solvents with aqueous buffer solutions, the papers being pretreated with the same buffers. The solvents tested included a range of the lower alcohols (methyl, ethyl, *n*-propyl, isopropyl, *tert*-butyl, with additions in some cases of higher alcohols), acetone, and methyl ethyl ketone; the buffers included oxalate, succinate, tartrate, citrate, maleate, carbonate, phosphate, and pyrophosphate.

Whilst these early tests were conducted under conditions which would now be regarded as primitive, some of the combinations showed promise. For example, systems comprising miscible alcohols and aqueous phosphate, tartrate, or pyrophosphate buffers gave generally improved chromatograms, often with small spots, and with indications of useful patterns of separation.

A Selection of Potentially Useful Systems

When the type 3 unit became available, a selection of the more promising combinations of miscible alcohols and buffers were re-examined with the improved means of control so afforded. This second screening revealed various

potentially useful systems, of which 10 examples are illustrated in Fig. 2.

Systems Nos. 1 to 7 are representative of the new group of buffered alcohol systems, whereas Nos. 8 to 10 are buffered phenol or phenol–cresol mixtures. The latter were included since it seemed likely that a solvent of phenolic character might be needed for the separation of serine and glycine. These patterns exhibit fair resolutions, ranging from 9 to 11 separated groups in the different alcoholic systems and from 10 to 11 groups in the phenolic systems. It should be emphasized that these illustrated patterns represented in most cases the results of only preliminary tests of the different systems, and there is no doubt that all the alcoholic systems could be adjusted and improved greatly by systematic modifications of the type to be described below.

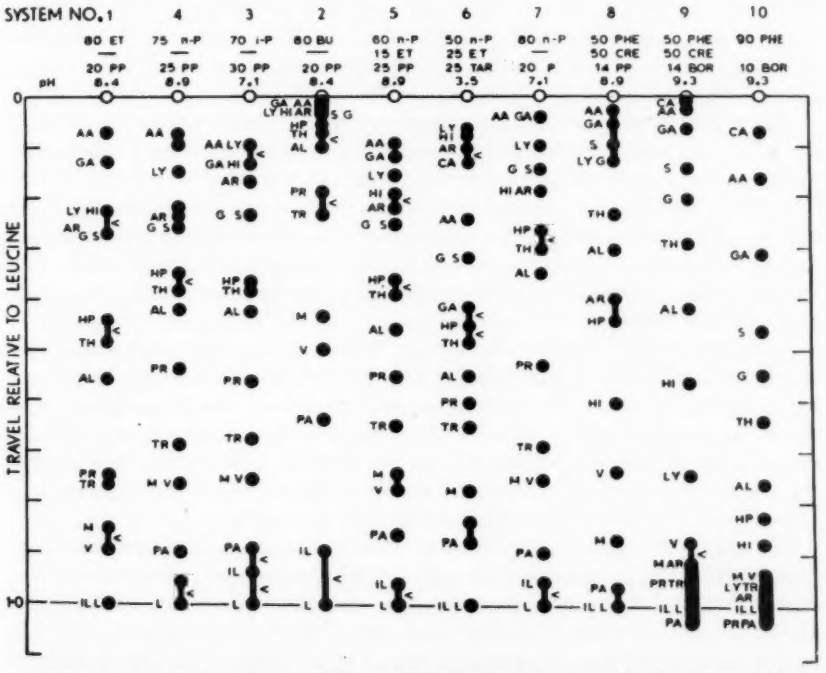

FIG. 2. A selection of potentially useful buffered systems which showed promising patterns of separation in preliminary tests. Details of these numbered systems are given in Tables I and II.

In considering the effects of different factors upon the alcohol – aq. pyrophosphate system it will be convenient to refer to two regions of the pattern of separation, viz. the back portion of the pattern, including the slow-moving amino acids (aspartic acid, cysteic acid, glutamic acid, lysine, histidine, arginine, serine, and glycine), and the forward portion, including the fast-moving amino

acids (hydroxyproline, threonine, alanine, proline, tryptophan, tyrosine, methionine, valine, phenylalanine, isoleucine, and leucine).

A comparison of the patterns of separation of the alcohol – aq. pyrophosphate systems (Nos. 1 to 5) shows that the particular alcohol used has little effect upon the order in which the different amino acids travel but it may influence markedly their spacing over the length of the chromatogram. With the ethanol – aq. pyrosphosphate system (No. 1) the forward portion of the pattern is somewhat contracted, especially the eight fastest-running amino acids (proline to leucine), which are crowded into a short length at the bottom of the chromatogram. It will be noted that large gaps occur along the chromatogram.

System No. 4 (propanol – aq. pyrophosphate) produced a more even distribution of the spots over the entire length of the chromatogram, the fast-running amino acids being relatively well separated. The positioning of the slow-running amino acids is also improved due to the decreased rate of movement of lysine. This is true to a lesser extent of the isopropanol – aq. pyrophosphate solvent (No. 3).

The butanol – aq. pyrophosphate system (No. 2) produced good separation of all but two of the fast-running amino acids (hydroxyproline and threonine) but the pattern shows severe crowding of the back portion.

With system No. 5 (propanol – ethanol – aq. pyrophosphate) improved spacing of the slow-running amino acids was obtained, combined with good separation of the fast-running amino acids. (This suggests that systems containing butanol and a lower alcohol might also be developed to separate both slow and fast-running amino acids.)

As regards other buffers which were tested in miscible alcohol systems, tartrate and phosphate deserve special mention. The patterns produced by a propanol – ethanol – aq. tartrate system and a propanol – aq. phosphate system are shown in Fig. 2, Nos. 6 and 7. The small compact spots in the tartrate system (No. 6) and the order of run characteristic of an acid pH showed promise; later a valuable pattern of separations based upon this system was evolved and this will be described in Part 4 of this series.

The propanol – aq. phosphate system (No. 7) produced, in general, compact spots but there was a marked tendency of the fast-running amino acids to form 'bearded' spots. However, useful systems containing phosphate buffers were developed and used at this stage (e.g. 26). Another such system (70 vol. *n*-propanol – 30 vol. aq. phosphate buffer at pH 6.0) was used successfully to separate N-ethylmaleimide complexes of sulphydryl-containing amino acids and peptides. The main weakness of the alcohol – aq. phosphate systems, namely, 'bearding' of fast-running amino acids, is completely absent from alcohol – aq. pyrophosphate systems, which are accordingly much preferred.

The three phenolic systems illustrated (Nos. 8, 9, and 10) all separated serine and glycine from one another, but in the pyrophosphate-buffered system (No. 8) glycine and lysine run together and arginine and histidine form 'streaky' spots. It should be pointed out that system No. 9 is that developed

by Levy and Chung (6) and used in their highly successful two-dimensional separations. No. 10 (phenol – aq. borate) represents a slight modification of a system described by McFarren (8) and this offers the advantage of separating cysteic acid and aspartic acid as well as serine and glycine.

Factors Influencing the Patterns of Separation in Propanol – Ethanol – Aq. Pyrophosphate Systems

The preliminary testing of the miscible alcohol – aq. pyrophosphate systems indicated that these gave separations which would be readily adjustable in response to variations in different conditions, as is suggested by the patterns in Nos. 1 to 5, Fig. 2. In the experiments now to be described the effects of systematic variation of the following factors were studied: (*a*) solvent composition, (*b*) pH of the pyrophosphate buffer, (*c*) additions of sodium chloride to solvent and paper, and (*d*) composition of the conditioning liquid.

(a) Influence of Solvent Composition

The rather complex effects of varying the proportions of propanol, ethanol, and pyrophosphate buffer solution may be seen from the patterns shown in Fig. 3 and Fig. 4 (in part). The three series of patterns in Fig. 3 show the effects of increasing the ethanol content at the expense of propanol, the water content being constant in each series. It is seen that addition of ethanol produces only minor changes in the order of run of the amino acids and that the main effect is the opening out of the back portion of the pattern with an improved separation of the slow-running amino acids.

The effect of varying the water content in the absence of ethanol is illustrated in Fig. 4 (Nos. 11, 4, and 14). A water content of about 25% appears to be nearly optimum since the back portion of the pattern is distributed over a maximum length and the faster-running amino acids are fairly evenly spaced. The addition of higher amounts of water leads to excessive movement of the pattern as a whole (No. 14) and if the water content is increased to 35% 'water-logging' effects are observed.

In the ternary mixtures (propanol – ethanol – aq. pyrophosphate) the optimum water content depends to a certain extent upon the proportion of ethanol present and the converse also is true. This is apparent from a comparison of the patterns in Fig. 3. Superior separations could be obtained with solvents containing either 20 or 25% water but those with 20% water required more ethanol for maximum resolutions. In fact, there was little to choose at this stage between the patterns produced with solvents containing the following proportions of propanol – ethanol – aq. pyrophosphate buffer: (*a*) 40:40:20; (*b*) 50:30:20; (*c*) 50:25:25. The resolutions in these respective systems (Nos. 13, 12, and 18) were 13–15 separate spots and the patterns of separations, although showing characteristic differences, were generally similar.

Variations in the proportions of propanol, ethanol, and water over the ranges tested produced only minor effects upon the pattern of separation of the fast-running amino acids.

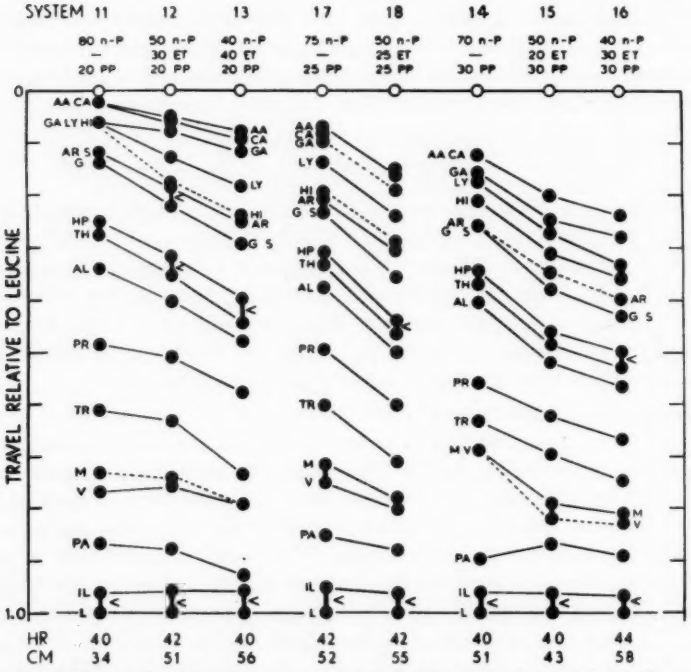
SYSTEM 11 12 13 17 18 14 15 16
80 n-P — 20 PP
50 n-P 30 ET 20 PP
40 n-P 40 ET 20 PP
75 n-P — 25 PP
50 n-P 25 ET 25 PP
70 n-P — 30 PP
50 n-P 20 ET 30 PP
40 n-P 30 ET 30 PP
TRAVEL RELATIVE TO LEUCINE
0
1.0
AA CA
GA LY HI
AR S G
HP TH
AL
PR
TR
M V
PA
IL L
HR 40 42 40 42 42 40 40 44
CM 34 51 56 52 55 51 43 58

FIG. 3. *n*-Propanol – ethanol – aqueous pyrophosphate systems. The influence of varying the proportions of propanol and ethanol in the solvent. Details of these numbered systems are given in Tables I and II.

(b) Influence of the pH of the Buffer

Systems Nos. 19, 20, and 21-A contained aq. pyrophosphate buffers at pH 2.0, 7.1, and 8.9 respectively. The patterns illustrate the generalization mentioned earlier that the order of travel of the neutral amino acids is little affected by changes in pH, while the positions of the acidic and the basic amino acids, respectively, are markedly affected in opposite senses. The pattern of separation of the acid pyrophosphate system, No. 19, appeared at first to indicate useful possibilities, especially for the separation of the basic amino acids, but in tests of numerous variants of the system it was not found possible to eliminate a number of serious weaknesses, especially the formation of streaky, diffuse spots and a low loading capacity for the amino acids. Furthermore, there was little evidence of adjustability of this system. In both neutral and alkaline systems, however (Nos. 20 and 21-A) small compact spots were obtained and improved resolution of the fast-running amino acids. With the increase in pH of the buffer from 7.1 to 8.9 an important opening out of the back portion of the pattern occurs, this being extended over a sufficient length to permit individual separation of the acidic and basic amino acids and serine + glycine (e.g. No. 21-

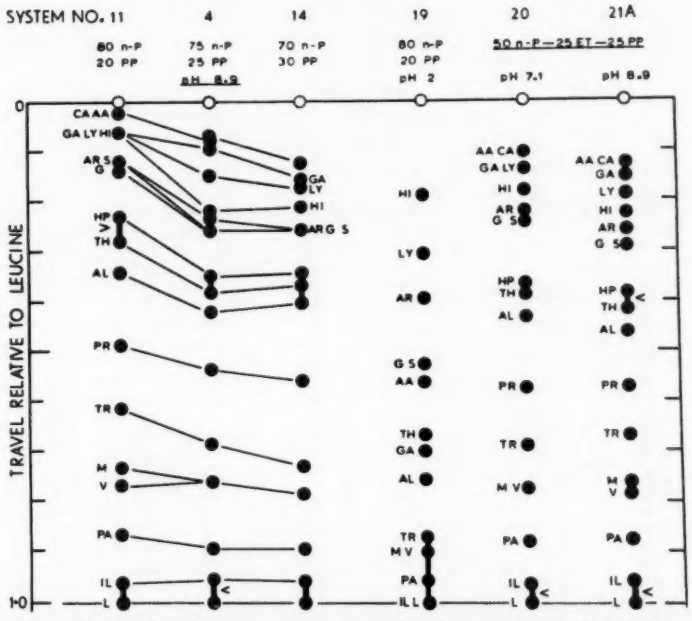

FIG. 4. *n*-Propanol – ethanol – aqueous pyrophosphate systems. The influence of varying the water content of the solvent and the pH of the pyrophosphate buffer. Details of these numbered systems are given in Tables I and II.

A). Chromatograms from systems containing buffers at intermediate pH values (e.g. pH 7.8, 8.1, 8.4) showed intermediate patterns with no advantages over that at pH 8.9. Changes over this narrow range of pH 7 to 9 do not influence significantly the forward portion of the pattern, but clearly a pH of the order of 8.9 offered more space for adjusting the positions of the slow-running amino acids.

(c) Influence of Additions of Sodium Chloride

The inclusion of sodium chloride as a useful constituent of propanol – ethanol – aq. pyrophosphate systems arose from the chance observation that the pattern was influenced by whether the pyrophosphate buffer had been prepared from the tetrasodium salt adjusted to a required pH with hydrochloric acid, or from the disodium salt adjusted with sodium hydroxide. The presence of chloride caused a relative acceleration of the basic amino acids (ornithine, lysine, and arginine, especially) in relation to neighboring amino acids, an effect which had already been remarked upon by other workers (9, 27). From this developed the addition of sodium chloride to these systems with the twofold object of controlling the positioning of these amino acids in the back portion of the pattern and at the same time of imparting immunity from the usual 'salt effect', i.e. disturbances of the chromatography from the presence of salts in

the solutions spotted on the starting line. Both objects have been attained but only after detailed studies of the influence of additions of sodium chloride to

TABLE I

Specification of systems

A. Alcoholic systems

System No.	Paper pretreated with buffer	Irrigating solvent: Alcohol, vol.	Aq. buffer, vol.	Water, vol.	NaCl,* g	Conditioning liquid: Alcohol, vol.	Water, vol.
1	A	Et, 80	A, 20		0.40	Et, 80	20
2	A	Bu, 80	A, 20		0.40	Bu, 80	20
3	C	*i*-P, 70	C, 30		0.18	*i*-P, 70	30
4	B	*n*-P, 75	B, 10	15	0.25	*n*-P, 75	25
5	B	*n*-P, 60 + Et, 15	B, 10	15	0.25	*n*-P, 75	25
6	D	*n*-P, 50 + Et, 25	D, 25	—	—	*n*-P, 75	25
7	E	*n*-P, 80	F, 20	—	0.84	*n*-P, 80	20
11	B	*n*-P, 80	B, 10	10	0.20	*n*-P, 75	25
12	B	*n*-P, 50 + Et, 30	B, 10	10	0.20	*n*-P, 75	25
13	B	*n*-P, 40 + Et, 40	B, 10	10	0.20	*n*-P, 75	25
14	B	*n*-P, 70	B, 10	20	0.30	*n*-P, 75	25
15	B	*n*-P, 50 + Et, 20	B, 10	20	0.30	*n*-P, 75	25
16	B	*n*-P, 40 + Et, 30	B, 10	20	0.30	*n*-P, 75	25
17	B	*n*-P, 75	B, 10	15	0.25	*n*-P, 83	17
18	B	*n*-P, 50 + Et, 25	B, 10	15	0.25	*n*-P, 83	17
19	I	*n*-P, 80	I, 20	—	0.14	*n*-P, 80	20
20	C	*n*-P, 50 + Et, 25	C, 10	15	0.25	*n*-P, 75	25
21	B	*n*-L, 50 + Et, 25	B, 10	15	0.25	*n*-P, 75	25
22	B	*n*-P, 50 + Et, 25	B, 10	15	0.53	*n*-P, 75	25
23	B	*n*-P, 50 + Et, 25	B, 10	15	1.05	*n*-P, 75	25
24	B	*n*-P, 50 + Et, 30	B, 10	10	0.25	*n*-P, 87	13
25	J	*n*-P, 50 + Et, 30	B, 10	10	0.25	*n*-P, 87	13
26	K	*n*-P, 50 + Et, 30	B, 10	10	0.25	*n*-P, 87	13
27	C	*n*-P, 70	C, 10	20	0.63	*n*-P, 89	11
28	C	*n*-P, 70	C, 10	20	0.63	*n*-P, 75	25
29	C	*n*-P, 70	C, 10	20	0.63	*n*-P, 65	35
30	B	*n*-P, 40 + Et, 40	B, 10	10	0.25	*n*-P, 89	11
31	B	*n*-P, 40 + Et, 40	B, 10	10	0.25	*n*-P, 85	15
32	B	*n*-P, 40 + Et, 40	B, 10	10	0.25	*n*-P, 75	25
37	B	*n*-P, 55 + Et, 22.5	B, 10	12.5	0.05	†	
38	B	*n*-P, 55 + Et, 22.5	B, 10	12.5	0.43	†	

B. Phenolic systems

System No.	Paper pretreated with buffer	Irrigating solvent: Phenol, g	Cresol, g	Aqueous buffer, ml	Conditioning liquid
8	B	50	50	B, 14	Water
9	G	50	50	G, 14	Water
10	H	90	—	H, 10	Water
33	H	93	—	H, 7	Water
34	H	85	—	H, 15	Water
35	B	93	—	B, 7	Water
36	G	93	—	G, 7	Water

*This refers to total NaCl, including that of the buffer, present in the solvent from 100 ml of components, not corrected for volume change.

†Conditioned by passing in an air stream which had been bubbled through a solution of *n*-P, 100 vol. + water, 15 vol.

TABLE II

Composition of buffers

Buffer	pH	Composition	Preparation
A	8.4	0.05 *M* pyrophosphate + 0.10 *M* sodium chloride	Tetrasodium pyrophosphate is adjusted with hydrochloric acid. Sufficient extra sodium chloride is added to give a total sodium chloride content of 0.10 *M* and the pH is adjusted again with sodium hydroxide
B	8.9	0.05 *M* pyrophosphate + 0.10 *M* sodium chloride	As for A above but see detailed preparation below*
C	7.1	0.05 *M* pyrophosphate + 0.10 *M* sodium chloride	As for A above
D	3.5	0.10 *M* tartrate	Tartaric acid is adjusted with sodium hydroxide
E	7.1	0.05 *M* phosphate + 0.10 *M* sodium chloride	The sodium dihydrogen phosphate and sodium chloride are dissolved together and the pH is adjusted with sodium hydroxide
F	7.1	0.10 *M* phosphate + 0.10 *M* sodium chloride	As for E above
G	9.3	0.06 *M* borate + 0.10 *M* sodium chloride	Boric acid and sodium chloride solution is adjusted with sodium hydroxide
H	9.3	0.06 *M* borate	Boric acid adjusted with sodium hydroxide
I	2.0	0.05 *M* pyrophosphate + 0.12 *M* sodium chloride	Tetrasodium pyrophosphate is adjusted with hydrochloric acid
J	8.9	0.05 *M* pyrophosphate + 0.20 *M* sodium chloride	As for A above
K	8.9	0.05 *M* pyrophosphate + 0.40 *M* sodium chloride	As for A above

*Preparation of the most commonly used buffer, B: To prepare 1 liter of buffer, 22.31 g of tetrasodium pyrophosphate ($Na_4P_2O_7.10\ H_2O$) is dissolved in 900 ml of water. To this is added 6.5 ml *N* HCl and 5.44 g sodium chloride. The volume is made up to 1 liter. The final pH should be 8.9 ± 0.05.

the solvent, on the one hand, and to the buffer solution used for impregnating the paper, on the other. Appropriate additions to both are required to avoid the formation of 'secondary fronts' on the chromatograms arising from the migration of sodium chloride (probably accompanied by water) from liquid phase to gel phase, or vice versa; these are eliminated when the irrigating solvent and the paper have been brought into an appropriate balance as regards sodium chloride content.

Apart from the specific accelerating effect of chloride ion upon the basic amino acids (which suggests that these migrate as salts and that the chlorides migrate more rapidly than the pyrophosphates) there is ample evidence that the addition of salt brings about other modifications of the pattern which are the result of changes in the distribution of water between the vapor, liquid, and gel phases of the system.

(i) Sodium Chloride Content of the Solvent

The forward portion of the pattern of separations is but little affected by varying the sodium chloride content of the solvent. A pronounced influence upon the back portion is illustrated in Fig. 5 (left) in which the positions of only the slow-running amino acids (cysteic acid to serine) are plotted, in terms of R_{serine} values (instead of the usual R_L values). It should be mentioned that two of the examples given — Nos. 37 and 38 — were obtained with systems not

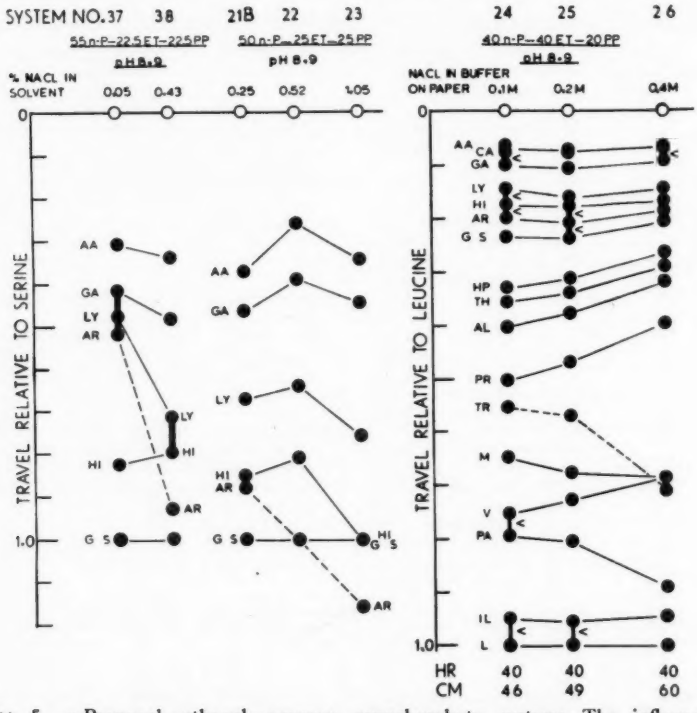

FIG. 5. *n*-Propanol – ethanol – aqueous pyrophosphate systems. The influence of varying the sodium chloride content of the solvent and of the paper. Details of these numbered systems are given in Tables I and II.

specified fully in Table I, since a different procedure for conditioning the unit was used but the variations in pattern are known to be attributable to the different sodium chloride contents of the solvent. The dramatic effects of increased chloride concentration may be seen by tracing the positions of the basic amino acids through this sequence of diagrams. (It is noteworthy that the imidazole nitrogen of histidine of which the migration rate is little altered would probably exist in the uncharged state in these systems; histidine has been found to be accelerated by chloride in more acid systems.)

(ii) Sodium Chloride Content of the Paper

In sharp contrast, variation of the sodium chloride content of the paper has only a minor retarding effect upon the back portion of the pattern (aspartic acid to serine) and no significant acceleration of the bases is evident, but the forward portion of the pattern is markedly affected. The patterns in Fig. 5 (right) for example, show considerable and progressive retardation of the sequence hydroxyproline to proline, as the salt content of the paper was increased. But amongst the fast-running amino acids, tryptophan and phenyl-

alanine are considerably accelerated and sometimes (not illustrated in the present experiment) methionine, but to a lesser degree.

The resulting rearrangement of the order and the positions of the fast-running amino acids is in all respects similar to that which results from an increased water content of the conditioning liquid (cf. Fig. 6). A tentative explanation

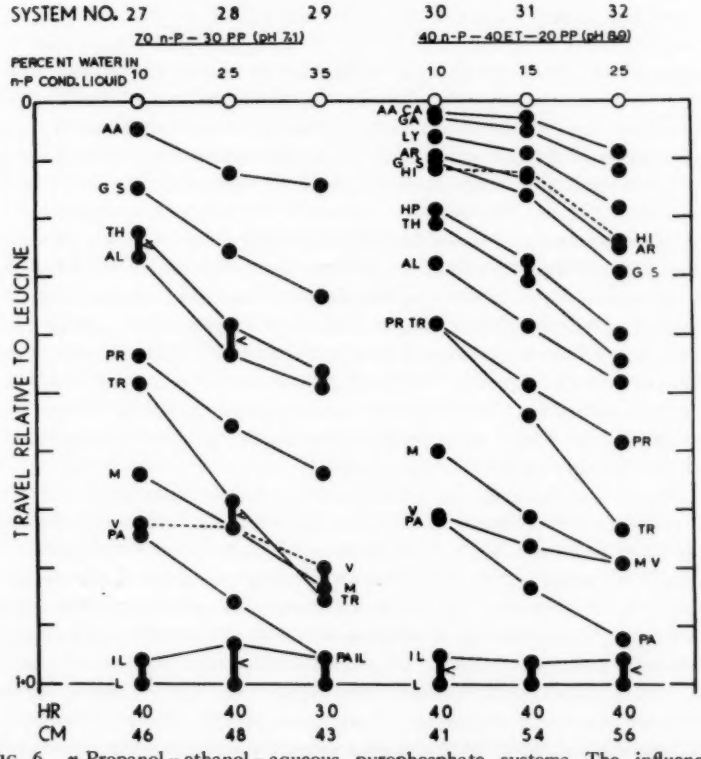

FIG. 6. *n*-Propanol – ethanol – aqueous pyrophosphate systems. The influence of varying the water content of the conditioning liquid. Details of these numbered systems are given in Tables I and II.

for the obviously complex effects of increased sodium chloride in the paper can be suggested in terms of the increased hydrophily which it would impart. In the upper region of the paper, nearer the solvent source, this would result in removal of water from the liquid phase by the gel phase with a consequent reduction in the rate of travel of the amino acids, especially the slow-running ones (cf. Fig. 4A); over the lower regions of the paper the lost water would be regained by uptake from the vapor phase and the final water content of liquid and gel phases toward the bottom would be expected to be increased by

increased salt content. It is thus possible to envisage longitudinal profiles of partitioning conditions which would result in the observed alterations in pattern.

(d) Influence of Composition of the Conditioning Liquid

It is often assumed, as was pointed out in Part 2 (1) that the objective of the preconditioning treatment should be to secure full equilibration of the vapor phase and the papers with the volatile constituents of the irrigating solvent. Early experiments with the type 3 unit showed that this was not necessarily the optimum initial state. It was found that improved chromatograms were obtained with propanol – ethanol – aq. pyrophosphate systems by omitting ethanol from the conditioning liquid, the spots being generally smaller, especially those of the fast-running amino acids. Other experiments with these systems confirmed the conclusions of McFarren (8) that the main beneficial effects of conditioning treatments stem from the adjustment of the moisture content of the system. It was shown, for example, that although poor chromatography (large diffuse spots and an adverse distribution) resulted from conditioning with pure water, much improved chromatograms resulted from the use of a water – ethylene glycol solution with an appropriately reduced water vapor pressure. However, conditioning with *n*-propanol–water solutions gave still superior chromatograms, indicating that water vapor is not the only volatile constituent worthy of control. (A limitation in using a single solution as the conditioning liquid, as is usually done, is that the vapor pressures of the constituents cannot be varied independently.)

The effects of increasing the water content of the *n*-propanol–water conditioning liquid in two different systems is shown in Fig. 6. The absolute rate of travel of all the amino acids is considerably increased, but not equally. This is shown by the considerable alteration in the forward part of the pattern; in both systems, tryptophan and phenylalanine, and to a lesser extent methionine, are accelerated more by increasing water in the conditioning liquid than are the neighboring amino acids. This results in a redistribution of the faster-running amino acids similar to that noted above when large amounts of sodium chloride were present in the paper. It would appear from Fig. 6 that optimum distribution is obtained in both systems when the conditioning liquid contains about 15% water.

It should be mentioned in this connection that it was impossible at this stage in the investigation to maintain close control of these features of the pattern which were most affected by changes in water vapor. During periods of uniform dry weather fair reproducibility of a desired pattern of separation could be obtained, but with the onset of hot humid weather there was increased variability, with a tendency for the chromatograms to be overrun and to show the defects similar to those obtained by the use of a conditioning liquid with excessive water. It became evident that under humid climatic conditions, an excess of water might be present in the filter papers when they were introduced

into the unit and that this excess was not removed by the conditioning procedure as used in the type 3 unit. In fact, as will be shown in Part 4, it was not until specific means were developed to take into account the variable amount of water imbibed in the chromatograms that adequate control of the system, independent of climatic conditions, was obtained.

Factors Affecting Patterns of Separation in Phenol and Phenol–Cresol Systems

In contrast to the sensitive miscible alcohol–buffer systems of high resolving power considered above, the different phenolic systems studied proved to be relatively insensitive to variations in many factors and this was coupled with a limited resolving power and adjustability. A phenol – aq. borate system of the type developed by McFarren (8) and Levy and Chung (6) was found to be superior to others tested for the resolution of some slow-running amino acids (cysteic acid, aspartic acid, glutamic acid, serine, and glycine) but no useful separation of the fast-running amino acids could be obtained with this or any other of the phenol and phenol–cresol systems tested.

As mentioned earlier, a simpler conditioning by means of a tray of pure water in the bottom of the unit was adequate for these phenolic systems. The patterns of separation proved to be only slightly influenced by changes in the water content of the irrigating solvent, increased water content accelerating the movement of all the amino acids and producing larger spots. Acceptable separations are illustrated in Fig. 7, Nos. 33 and 34, but system No. 10 (Fig. 2) was preferred because the spots were appreciably smaller.

Trials of the pyrophosphate buffer (pH 8.9) used so successfully in the miscible alcohol systems were less successful with phenolic solvents. For example, although serine and glycine were separated in system No. 35 (Fig. 7), serine ran with glutamic acid, and glycine with lysine. It is noteworthy that the basic amino acids travel much more slowly in phenolic systems with pyrophosphate as compared to borate buffer. The patterns of separation obtained with cresol – phenol – aq. buffer systems as illustrated by Nos. 8 and 9 (Fig. 7) offered no obvious advantages.

Additions of sodium chloride to phenolic systems conferred no benefits; the presence of sodium chloride in phenol – aq. borate systems resulted in some minor chromatographic defects e.g. a characteristic distortion of aspartic acid and an increased tendency of all the spots to become streaky. The presence of 0.5 *M* sodium chloride in the amino acid solution spotted on the chromatogram, however, does not impair the resolution.

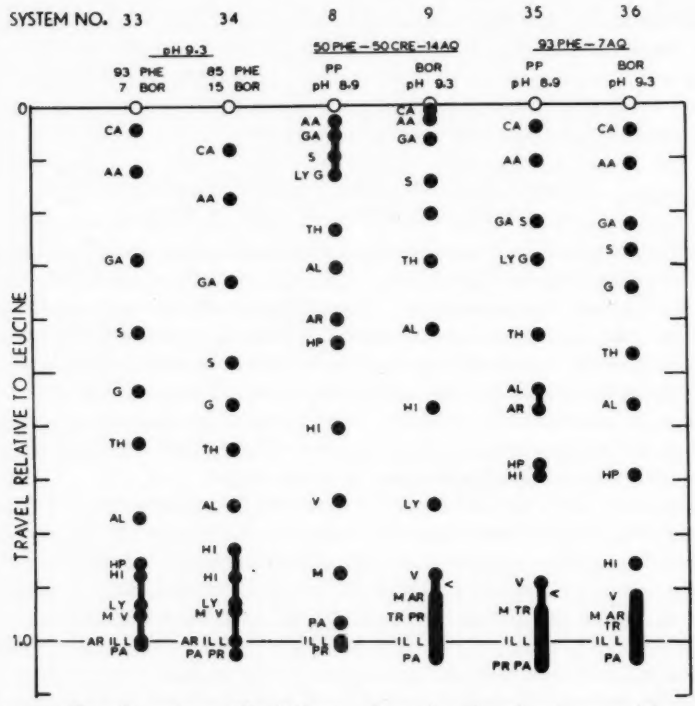

FIG. 7. Phenolic systems. The influence of varying the solvent composition, buffer ion, and pH. Details of these numbered systems are given in Tables I and II.

Discussion

The patterns of separation of amino acids reported for many one-dimensional systems were surveyed and the possibilities were then explored of securing improved resolution by the use of a neglected group of systems, namely miscible alcohols used with appropriate aqueous buffers, the papers being pretreated separately with the buffers. Systems of this type showed promise in preliminary tests; they were found to be highly adjustable in the sense that many characteristics of the chromatographic patterns, including the size and spacing of many of the separated spots and, for some groups of amino acids, even their order in the pattern could be varied by controlled alterations in the conditions.

One family of systems of this type, based on *n*-propanol, ethanol, and aqueous pyrophosphate buffers, with added sodium chloride, was selected as an example for systematic study of the influence of variations in the composition of the solvent, the pH of the buffer, the sodium chloride content of the solvent and of the buffer used for impregnating the paper, and finally the composition of the vapor phase in the chromatographic unit.

The extensive observations made upon the *n*-propanol – ethanol – aq.

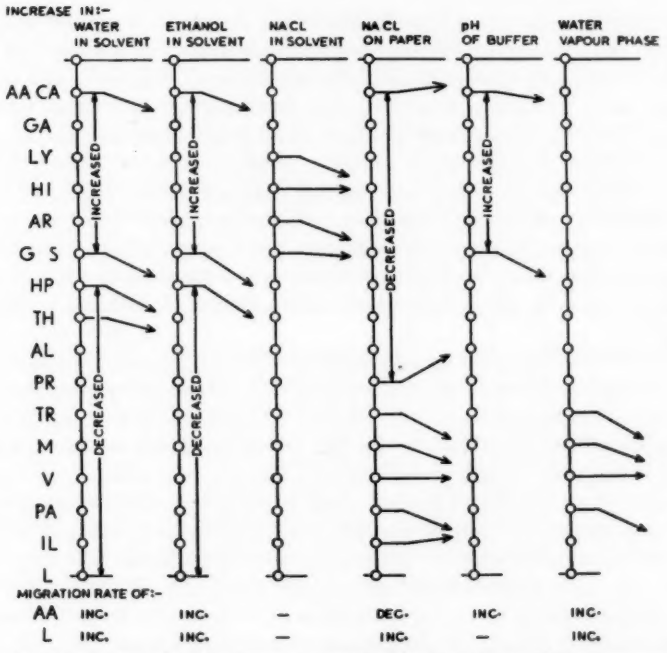

FIG. 8. Propanol – ethanol – aqueous pyrophosphate systems. Diagram summarizing the influence of different factors which may be used to adjust and control the patterns of separation.

pyrophosphate systems have been summarized schematically in Fig. 8. Three main features may be seen to be controllable by appropriate variations in the different factors studied. The first feature, controllable jointly by the concentrations of water and of ethanol in the solvent, and by the pH of the buffer, is the length of the chromatogram over which the slow-running amino acids (aspartic acid to glycine + serine) are distributed; it proved desirable that this length be increased to a maximum in order to provide space for separating the individual members of this group. The second feature is the positioning of lysine and arginine amongst the neighboring amino acids in the back portion of the pattern, this being controlled dramatically by the sodium chloride content of the solvent. The third main feature is the positioning of tryptophan, methionine, and phenylalanine amongst their neighbors in the forward portion of the pattern, which is controlled by varying either the sodium chloride content of the paper or the water content of the conditioning liquid, both factors operating apparently by leading to increased water content of the stationary gel phase.

It has been possible to offer at least partial explanations for some of these effects in terms of admittedly somewhat speculative mechanisms but, for

present purposes, the main significance of the results summarized in Fig. 8 is the rather detailed picture they afford of the interplay of a variety of factors upon the functioning of this group of chromatographic systems. This picture is probably more comprehensive than that available for any other group but there are indications that most of the systems in common use may be subject in varying degree to adjustment by the controlled variation of such factors.

As a result of these studies it was possible to specify conditions which resulted in approximately optimum resolutions with the propanol – ethanol – aq. pyrophosphate system, the number of separated spots varying from 13 to 15 from a mixture of 18 amino acids. The desired pattern of separation, with only minor differences, could be obtained by the use of solvents of different composition, as follows:

50 vol. *n*-propanol – 30 vol. ethanol – 20 vol. aq. pyrophosphate
40 vol. *n*-propanol – 40 vol. ethanol – 20 vol. aq. pyrophosphate
50 vol. *n*-propanol – 25 vol. ethanol – 25 vol. aq. pyrophosphate

Of these the 50 vol. *n*-propanol – 25 vol. ethanol – 25 vol. aq. pyrophosphate buffer system, used as specified in system No. 21, was selected as best. The high resolving power of this system made it possible to propose a fairly satisfactory complementary pair of one-dimensional systems capable of separating all the amino acids present in acid hydrolyzates of proteins. The *n*-propanol – ethanol – aq. pyrophosphate system usually separated all these amino acids except the pairs aspartic + cysteic acids and serine + glycine, and these pairs could be resolved cleanly in the phenol – aq. borate system (No. 10). Apart from the fact that the separation of histidine and arginine was not dependable and that there were objections on analytical grounds to phenolic solvents, these complementary systems were used successfully for protein analysis.

It should be emphasized finally, that although approximately optimum separations were obtainable in the *n*-propanol – ethanol – aq. pyrophosphate system with fair reproducibility over periods of several weeks, there was evidence of the existence of at least one remaining major source of variability which had not been brought under adequate control. This was traced ultimately to variations in the initial moisture content of the filter paper corresponding to differences in the ambient humidity. In the succeeding communication a simplified procedure will be described for the preconditioning of chromatographic systems which includes control of this remaining factor.

Acknowledgments

The authors wish to thank Mrs. E. Tigane and Dr. C. K. Harris for much valuable assistance and advice. They are pleased to acknowledge the generous support of the work by grants from the National Research Council of Canada, the National Cancer Institute of Canada, and, in the initial stages of the work, the J. P. Bickell Foundation.

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QUANTITATIVE CHROMATOGRAPHIC METHODS
PART 4. STABILIZED CHROMATOGRAPHIC SYSTEMS OF HIGH RESOLVING POWER FOR AMINO ACIDS[1]

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With appended note on

MOISTURE RELATIONS OF CHROMATOGRAPHIC PAPERS

by

C. S. HANES AND D. D. WOOD

Abstract

The development of two paper chromatographic systems of high resolving power for the amino acids is described. Both systems are based on buffered miscible solvents used with buffer impregnated papers. By selection and control of the conditions of operation, the patterns of separation of these systems have been adjusted to yield maximum combined resolution when they are used together as a complementary pair. The general problems and possibilities of attempting to select and refine detailed features of paper chromatographic patterns seem to be well illustrated by the development of these two systems.

Introduction

In the preceding studies (1, 2) a variety of means were explored for improving the separation of amino acids on one-dimensional paper chromatograms. The primary object was to provide an improved basis for the quantitative analysis of the amino acids, but it has been kept in view that the improvement of chromatographic resolutions in one-dimensional irrigations might contribute also to improved two-dimensional separations for which there is an increasing need in some fields, e.g. for separating complex mixtures of peptides in connection with constitutional studies on the proteins (3, 4, 5).

In the preceding communication, Part 3 of this series (2), emphasis was placed upon a neglected category of system, namely, buffered water-miscible solvents used with papers impregnated separately with the same buffers. One family of such systems, to be referred to now as P-E-PP systems (based on *n*-propanol, ethanol, and aqueous pyrophosphate buffers) was chosen for a systematic study of the effects of several variables upon the pattern of separations. As a result, it became possible to adjust and control at will many features of the chromatograms and to define conditions which led to considerably improved resolutions. In the optimum pattern of separations obtained with the P-E-PP system, 14 of the amino acids present in acid hydrolyzates of proteins could be separated for individual analysis and the remaining four were

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present as two unresolved pairs. Since both these pairs could be resolved in a second one-dimensional system (phenol – aqueous borate buffer) it could be claimed that an early objective had been attained of evolving a complementary pair of one-dimensional systems capable of separating the common amino acids.

While these systems, operated as described in Part 3, have been used successfully in connection with various problems, they retained evident weaknesses whose elimination has been the object of subsequent studies. In particular, the P-E-PP system still showed some variability which made it impossible to rely upon obtaining all the separations characterizing its optimum pattern. There were indications that the remaining variability reflected failure of the usual methods for the preconditioning of chromatographic systems, even with the efficient equilibration equipment provided in the type 3 unit, to compensate adequately for differences in the amount of moisture imbibed in the chromatographic papers. Furthermore, it was desirable, for routine use, to simplify the procedures for controlling the operation of the new chromatographic systems. Finally, it was hoped, if possible, to replace the phenol – aqueous borate system referred to above by one which would be free from certain analytical objections to phenolic solvents (to be discussed in Part 6 of this series) and which might at the same time supplement the P-E-PP system more effectively by extending the range of separable amino acids. In particular, it was hoped that a new partner for the P-E-PP system might permit the separation and accurate determination of asparagine and glutamine in cell extracts or amongst the products of enzymic hydrolysis of proteins and peptides.

Practical solutions to these different problems will be described in the present communication, as well as the simplified methods of operating selected stable systems of high resolving power which are now in use. The dual objectives of improved stability coupled with simplified equipment and procedures have resulted mainly from a novel approach to the preconditioning of chromatographic systems; this compensates for the wide variations in the moisture content of the filter papers which are introduced into a chromatographic system, depending upon the prevailing atmospheric humidity. The desired extension in the range of separable amino acids has been achieved by the development of a second buffered water-miscible system, as a partner for the P-E-PP system; this is based upon *n*-propanol, ethanol, and aqueous tartrate buffer and will be referred to as the P-E-T system.

In Fig. 1 are shown the typical patterns of separation which are now obtained reproducibly in the P-E-PP system (chromatogram 1A) and the P-E-T system (chromatogram 1C) as operated under conditions adjusted to give maximum mutual supplementation of the resolving capacities of these two systems. The full potentialities and limitations of these selected systems as applied to the separation and analysis of complex mixtures of amino acids will be considered critically later, but it may be stated that they approach an almost ideal complementary pair of one-dimensional systems. A major remaining deficiency is that, although glycine and serine are separated sufficiently in the

P-E-T system to permit an estimate to be made of the proportions of these two in a double spot, this estimate is subject to relatively large error. Accordingly, for a closer analysis of glycine and serine, use has still to be made of a phenolic solvent.

The separation of this one pair is effected in a manner illustrated also in Fig. 1. An area of the standard P-E-PP chromatogram, indicated in 1A, is cut out and is irrigated again for a short time by ascending chromatography in a solvent of phenol – aqueous pyrophosphate buffer, with the result shown in chromatogram 1B. This procedure has been found to reduce considerably the adverse effects of phenol upon the recovery values (apparently owing to the shortness of the time and the distance of travel involved) and it limits the use of phenol to this single pair of amino acids.

Apart from the inconvenience of this extra operation for the analysis of glycine and serine, the combination of the two systems can be relied upon to permit the separation and analysis of all the common amino acids, including glutamine and asparagine, with the exception of tryptophan, for which special procedures to be referred to in Part 6 of this series are available.

The purpose of the present communication is to specify the optimum conditions for operating the P-E-PP and the P-E-T systems and to describe briefly also the experimentation leading to the selection of these particular conditions. The observations will serve to illustrate the manner in which controlled adjustments of chromatographic patterns are attainable with the simple means provided in the type 4 chromatographic unit; the application of similar methods to the separation of other groups of compounds will be described in subsequent communications.

Equipment, Materials, and General Procedures

Equipment

*The Type 4 Chromatographic Unit**

The procedures to be outlined involve the use of the type 4 chromatographic unit, maintained at 34° C in a constant temperature cabinet. This equipment has been described in Part 2 (1).

The type 4 unit, like the type 3, includes the porcelain solvent trough designed to irrigate two chromatograms at a time, these being provided with stitched-on 'feeder wicks' to regulate the rate of flow of the solvent down the chromatogram.

The main feature of the type 4 unit distinguishing it from earlier designs is the arrangement for the preconditioning of the closed system. An external receiver tube, normally stoppered, is provided near the top of the unit and into this are pipetted small volumes of water and other liquids required to bring the closed system to a specified standard state. The added liquid flows down to a small paper evaporator inside the unit and, by operating a motor-driven fan

*This equipment is available from New Way Laminates Ltd., 29 Portland Street, Mimico, Toronto (14).

for a short period (normally 30 minutes), the liquid is evaporated and distributed rapidly between the vapor phase and the suspended chromatographic papers in the closed unit; the mass of the paper in the evaporator itself is small compared with that of the two chromatograms (about 4 g as compared with 70 g). It is to be noted that there is no provision in the type 4 unit for the continuous exposure of any pool of 'conditioning liquid' within the unit.

Equipment for Estimating Moisture Content of Chromatograms

One method for estimating the moisture content of chromatograms to be mentioned below involves the weighing of reference chromatograms (of known dry weight and made of the different buffered papers used) which are kept for this purpose exposed to the same atmospheric conditions as the actual chromatograms. For protection from dust, these are kept suspended in a screened-in cupboard which contains also a simple balance (accurate to 20 mg); this is mounted with a hanging counterpoised clip to which the chromatograms are transferred conveniently from their racks for rapid weighing.

An alternative method, now used as a routine in this laboratory, is to deduce the moisture content of the chromatograms from the relative humidity of the ambient atmosphere. This is read from a hair hygrometer of good quality, which is suspended in that part of the laboratory in which the chromatograms are normally spotted and exposed to the laboratory atmosphere during the period of an hour or longer before they are placed in the units. The setting of the hair hygrometer is checked once a month against a sling psychrometer. Hair hygrometer (Model PH/1 of Negretti and Zambra, London, also Toronto 14, etc.) has proved satisfactory.

Auxiliary Chromatographic Jar for Separating Serine and Glycine

It was mentioned above that one method for separating glycine and serine is to cut out an area from the finished P-E-PP chromatogram and to subject it to a second short irrigation in the reverse direction by ascending chromatography, using a phenol–aqueous pyrophosphate solvent (described below under Chromatographic Solvents). This is carried out at room temperature in a rectangular glass jar ($3\frac{1}{4} \times 8 \times 12\frac{1}{2}$ in. high) provided with a glass lid, the solvent forming a pool in the bottom of the jar. Inside the jar stands a loose support which consists of two upright glass rods, joined by an adjustable cross-bar of thin stainless steel, bent to clip over one of the glass rods at each end. The small chromatogram, normally about 3×8 in., carrying the unresolved glycine + serine spots near the bottom is clipped to the cross-bar, which is adjusted in height to allow the edge of the paper to dip into the solvent. No rigorous preconditioning is needed but under dry atmospheric conditions (e.g. relative humidities below about 40%) it is advantageous to expose the papers to a moist atmosphere for 15 minutes or so beforehand. The irrigation is allowed to proceed for about 7 hours. The cutting of the appropriate area for the auxiliary chromatogram will be described under Detection of Amino Compounds on the Chromatogram.

MATERIALS

Chemicals

n-Propanol (Reagent Grade), tetrasodium pyrophosphate decahydrate (Analar), tartaric acid (Analar), phenol (Analar), and silica gel (self-indicating, 6–20 mesh) were used as purchased from British Drug Houses (Canada), Toronto 14.

The purest grade of '95%' ethanol (rectified alcohol, 65% 'over proof') was used, the water content of 5.5 ± 0.5 vol.% being confirmed by hygrometer on each fresh batch.

Buffer Solutions

Buffer solution 1 (pyrophosphate, pH 8.9).—The pyrophosphate buffer (0.05 *M*, containing 0.12 *M* sodium chloride) is prepared as follows: 89.3 g tetrasodium pyrophosphate decahydrate and 26.55 g sodium chloride are dissolved in ca. 3800 ml water; 22.0 ml *N* hydrochloric acid is added. The pH (by glass electrode) should be at pH 8.90 ± 0.03; if not, it is adjusted with hydrochloric acid or sodium hydroxide. The volume is brought to 4000 ml with water. This solution contains 0.70 g sodium chloride per 100 ml (including that formed from the added hydrochloric acid).

Buffer solution 2 (tartrate, pH 3.4).—The tartrate buffer (0.10 *M*, pH 3.4) is prepared as follows: 60.0 g tartaric acid is dissolved in 3800 ml water; this is adjusted to pH 3.40 ± 0.03 with 5 *N* sodium hydroxide of which approximately 84 ml is required. The volume is adjusted to 4000 ml with water.

Chromatographic Solvents

In most of the solvents to be mentioned subsequently, the mixing of the buffer solutions, water, and alcohols (with extra sodium chloride in the case of P-E-PP solvents), results in the precipitation of small amounts of buffer salts; the volumes of buffer solutions specified have been adjusted to avoid an excessive precipitation with risk of differential removal of one species of buffer salt. The most satisfactory standard procedure for preparing a chromatographic solvent, normally 2 l., is to dissolve the sodium chloride (where specified) in the combined volumes of aqueous buffer solution and water; the specified volumes of the alcohols are then added and the whole mixture, contained in a stoppered Pyrex bottle, is brought to about 35–40° C by allowing it to stand in warm water. The bottle is then skaken vigorously and placed on the shelf provided in the constant temperature cabinet at 34° C—cf. (1). After a few hours (e.g. overnight) the supernatant solution can be withdrawn for use without filtration, the precipitate adhering as a crust to the bottle. This procedure ensures that the solvent will be saturated, but never supersaturated, with respect to buffer salts; the latter is to be avoided since it may affect the flow rate due to clogging of the feeder wick with crystals.

While the compositions of many different P-E-PP and P-E-T solvents will be indicated in what follows, it is proposed to specify here the compositions of only the two solvents selected as optimum for these respective systems.

Solvent for the P-E-PP system.—Two liters of the selected P-E-PP solvent (50 vol. *n*-propanol – 25 vol. ethanol – 25 vol. aqueous pyrophosphate buffer, with added sodium chloride) is prepared as described above from the following components: 200 ml buffer solution 1 (pyrophosphate), 300 ml water, 4.8 g sodium chloride, 500 ml 95% ethanol, 1000 ml *n*-propanol. The total sodium chloride content of this solvent is 3.1 g/liter.

Solvent for the P-E-T system.—Two liters of the selected P-E-T solvent (62.5 vol. *n*-propanol – 37.5 vol. aqueous tartrate buffer) is prepared likewise from the following: 750 ml buffer solution 2 (tartrate), 1250 ml *n*-propanol.

Auxiliary phenol – aqueous pyrophosphate solvent.—This solvent, used for separating serine and glycine by ascending chromatography applied to an excised area from the P-E-PP chromatogram, is prepared without the above precautions. It is made simply by adding 100 ml of buffer solution 1 (pyrophosphate) and 30 ml water to 400 g phenol. About 200 ml of this solvent is placed in the bottom of the glass jar used for this operation and is changed about once a month.

The Chromatograms

Details of the preparation of the chromatograms have been given in Part 2 of this series (1) and these require only a few additional comments in the present context.

As in the earlier work, the finally selected P-E-PP and P-E-T systems are based on the use of Whatman No. 3 filter paper. This is now purchased in sheets 29½ × 11 in., cut with the long dimension parallel to the machine direction of the paper. (Although this paper has a 'machine direction', it is not Grade No. 3 MM paper, which has been found to give different and, in these systems, inferior patterns of separation.) Recent experiments which have been possible as a result of the kind co-operation of Messrs. W. R. Balston Ltd., Maidstone, Kent, and Messrs. H. Reeve Angel and Co., London and New York, suggest that it may be possible to specify in the near future a modified type of Grade No. 3 paper which, in addition to exhibiting a low ninhydrin 'blank' value, has a special texture which permits further improvement in the resolutions obtained in these buffered miscible systems.*

The papers for use in the P-E-PP and P-E-T systems are impregnated with buffer solutions 1 (pyrophosphate) and 2 (tartrate), respectively, described above.

The chromatograms are provided with feeder wicks stitched across the top by sewing machine. The sewing should be done carefully, with uniform tension, and with fine needle and thread to cause minimum damage to the paper. The choice of type of paper for the feeder wick will be discussed separately under Adjustment of Rate of Flow of Solvent, below. Each chromatogram is provided with an absorption pad, snugly enclosed in a polyethylene wrapper, and clamped across the bottom. Details of dimensions, procedures for impregnation

*This new improved paper (to be referred to as Whatman No. 3 HR) is now available, cut in sheets 29½ in. × 11 in. for this work (cf. note in Part 2 (p. 133)).

of the papers with buffer solutions, attachment of wick and pad, and suspension of the chromatograms in the type 4 chromatographic unit have been given in Part 2 (1).

In the chromatograms to be illustrated, the amino acid solutions were applied from a micropipette delivering about 5 μl. The test solutions used most often contained 20 (or 22) amino acids, each present in a concentration of 0.02 *M*, so that the 'singly loaded' spot contained 0.1 μmole of each amino acid or 2 μmoles total amino acids. The effect of increased loading was tested as a routine on all chromatograms by 'multiple-spotting', spots consisting of single, double, treble, and often quadruple applications, with drying between; these loadings correspond to 0.1–0.4 μmole of each amino acid.

General Procedures

Preconditioning of Chromatographic Systems

The chromatographic units are left open for ventilation in the constant temperature cabinet after use so that each new pair of chromatograms is introduced into a dry unit which is then closed. The object of the preconditioning procedure is now to bring this closed system to a specified optimum initial state as regards its content of water and of any other desired volatile constituent before the solvent is added to the trough to commence the irrigation. The main variable is the amount of water present as imbibed moisture in the pair of chromatograms introduced into the unit. Under the range of conditions encountered in this laboratory, for example, the moisture content of the chromatograms varies from about 2 g in the driest winter periods to 10 g during the most humid spells of summer weather (or from about 3 to 15 g if the paper absorption pads are included). By comparison, variations in the amount of water vapor in the 56 liters of air in the 'dry' box are small, ranging from 0.2 to 1.2 g of water (as calculated from observed relative humidity values in the units) and can be neglected. The water content of the absorption pads can be neglected also when the pads are snugly wrapped in polyethylene sheeting since this effectively isolates the pads so far as exchange of water vapor is concerned (cf. 1, Fig. 9). The problem is then to estimate and to allow for the variable amounts of water present in the two chromatograms which are introduced into the unit.

The best approach may vary with the climatic setting and with the available facilities of any particular laboratory. For example, if the humidity of a laboratory were sufficiently constant, due either to natural conditions or artificial control, it might be found that the water content of the papers would remain within (say) $\pm$ 1 g of some mean value and that the addition of a standard volume of water (plus other specified liquid) would bring the system sufficiently close to a specified initial state. Air-conditioning of the laboratory would be a great convenience in this respect and it might result in slight improvements in the chromatography (for reasons which will be mentioned later) although it would not eliminate the need for further additions of water

and, of course, of other volatiles found desirable in particular chromatographic systems.

A possible alternative to controlling the humidity of the whole laboratory would be to expose the papers to an atmosphere of controlled humidity in a small cabinet. After preliminary tests, this method was rejected on the grounds that it constituted an additional operation requiring extra time and equipment, and that the final transfer of the conditioned papers to the chromatographic units without considerable changes in water content was by no means easy. The approach finally adopted is simpler and more convenient. It is to ensure that the papers as introduced into the chromatographic units are in a state of approximate equilibrium as to moisture content with the ambient (uncontrolled) humidity of the laboratory. This makes it possible to estimate the water content of the chromatograms by either of the two following methods:

(1) Reference chromatograms, which are also in equilibrium with the ambient atmospheric humidity, but of which the dry weights are known, are weighed at the time the actual chromatograms are to be placed in the unit. For this purpose reference chromatograms of the different buffered papers are kept exposed to the atmosphere in the screened-in cupboard described above.

(2) The relative humidity of the atmosphere is determined by sling psychrometer or calibrated hair hygrometer. From this value the moisture content of the pair of chromatograms is deduced from data established separately relating the relative humidity and the moisture content of the type of buffered chromatogram in question.

Both of these methods have been used throughout the extreme range of humidities (from about 10 to 80% relative humidity) experienced in this laboratory and have been found to be adequate for the purpose and to give estimates which are in substantial agreement. The second method, which requires only a reading of the ambient humidity, is more convenient, once the necessary moisture relations have been established. It may be noted that the first method may be used to accumulate the data needed for operating the second method if the relative humidity readings are made whenever the reference chromatograms are weighed.

A study of the moisture relations of unbuffered and variously buffered chromatograms is described in the Note appended to this communication. From this study were obtained the data of Table I relating relative humidity and moisture content of pairs of chromatograms of the standard size of Whatman No. 3 paper, unbuffered, and also impregnated with buffer solutions 1 and 2, as used in the selected P-E-PP and P-E-T systems, respectively.

These data, as will be seen later, permit the translation of any ambient humidity reading into the volume of water which must be added to the closed chromatographic unit to bring the total initial water content to the optimum value for the particular system.

Under very humid conditions, the moisture contained in the two chromatograms may exceed the recommended total initial amount. In this case, en-

countered here only rarely, the excess water is absorbed by anhydrous silica gel, which is packaged for the purpose in tubular sacs of fiber-glass screening, in 5-g and 10-g portions which absorb, respectively, 1 g and 2 g water. An appropriate number of these sacs is suspended in the lower part of the chromatographic unit; these remove the excess water effectively during the usual 30-minute period of fanning.

A further point in relation to moisture control deserves mention. The use of a warm air blower to dry the spots of solutions applied to chromatograms (e.g. between successive applications) may drive off as much as 3 g of water from a single chromatogram when the ambient humidity is high. When such a dryer is used, a full hour should be allowed before the chromatograms are placed in the unit, to permit them to regain moisture to something approaching the equilibrium state.

Adjustment of the Rate of Flow of Solvent

With the improved reproducibility resulting from the new method of preconditioning, the importance of controlling the rate of irrigation of the chromatograms was re-emphasized as a factor affecting not merely the distance of travel of the amino acids, but also the perfection of the resolutions and the accuracy of ultimate analysis. Examples will be presented which illustrate the value of an easy and flexible means for adjusting the flow rate of the irrigating solvent.

The rate of supply of solvent to the chromatogram is regulated by the type of paper used for the feeder wick and by the effective length of this wick adjusted by the means described in Part 2 (1).

It is unfortunately not yet sufficient to specify a desired flow rate for a given system by specifying merely the effective length and the grade of paper for the feeder wick because of batch-to-batch variability in the porosity and other physical properties of different batches of the grades of paper used both for the wick and the chromatogram proper. Instead, the desired rate of solvent flow must be defined in terms of the distance of travel of some particular compound during a stated period of irrigation, e.g. a 22- to 24-in. travel of leucine in 40 hours. Some guidance will be given in connection with the P-E-PP and P-E-T systems as to the range of wick lengths which have been found to give the desired rates of flow with different batches of Grades No. 1 and No. 3 papers. While it is relatively simple to establish the correct length of wick and volume of solvent to be used when work is begun with a new batch of paper (for either the wicks or chromatograms) it is desirable to purchase ample stocks of paper for both purposes at one time to avoid the need for too frequent calibrations of this kind.

It is appropriate to mention finally a number of specific factors which can cause irregularities in flow rate, namely, failure to cut the feeder wicks always in the same 'machine direction', excessive squeezing of the paper between the support rods, improper seating of the support rods on the rests, failure to

prevent the wick from approaching the wall of the solvent trough (thereby shortening the capillary path), unlevel mounting of solvent trough or whole chromatographic unit, and failure to ensure good capillary connection between absorption pad and the bottom of the chromatogram across its full width. Even when all these precautions are taken, there may still be observed appreciable variations in flow rate and in the movement of solutes, but these are much reduced and rarely detract from the usefulness of any chromatogram; they are perhaps attributable in the main to inequalities in the paper structure.

Detection of Amino Compounds on the Chromatogram

The photographs used for illustrating this communication have been taken by light transmitted through the chromatograms after a dipping treatment in a ninhydrin reagent which leads to an intense development of Ruhemann's purple. This treatment was based upon the procedure of Roland and Gross (6) developed for the quantitative determination of the amino acids by direct photometry on the paper. This intense coloration of the chromatograms has the advantages for present purposes of making visible the extreme limits of the different spots and hence of emphasizing any defects in the resolutions and, owing to its greater uniformity as compared with the more usual spraying treatment, it has facilitated the photography.

The dip reagent used by Roland and Gross contained only ninhydrin, acetic acid, and acetone. This has been modified by including collidine, which causes differential coloration to be exhibited by some of the amino acids (7). This effect has been especially advantageous in studying the resolutions of aspartic and cysteic acids, which stain bright blue and purple, respectively, of the basic amino acids amongst which histidine gives a grey-blue color, and of the serine and glycine pair of which the latter gives a grey color. These differences in hue have made it possible frequently to delineate clearly the boundaries between contiguous spots of these particular pairs of amino acids which have been the most difficult to resolve. Occasionally boundaries made visible by these color differences, which are not evident on the photographs, have been indicated by pencilled lines. Similarly the positions of proline, hydroxyproline, and asparagine are indicated in pencil since these areas are stained in various yellow hues which are invisible on the photographs.

The procedure followed for the ninhydrin treatment of the chromatograms for photography is as follows:

(*a*) The reagent for the dipping treatment is prepared freshly for each batch of chromatograms from 250 mg ninhydrin, 10 ml collidine, 25 ml glacial acetic acid, and 965 ml acetone. If no more than six to seven chromatograms are to be treated, one-half of the above volume is sufficient.

(*b*) The chromatograms are passed rapidly through this reagent contained in a flat dish, and are hung for 1–2 hours at room temperature under the vapor hood. By this time the positions of nearly all spots are discernible.

(*c*) The chromatograms are then suspended in the dark for a further 15–18

hours still at room temperature. By this time nearly full development of the color will have occurred. (The color development was occasionally accelerated by suspending the chromatograms for 1 hour in the constant temperature cabinet at 34° C.)

While this procedure has been much used for qualitative purposes, the dip reagent has been modified for quantitative work, as will be described in Part 6 of this series (8).

Reference should be made to the procedure used for localizing the position of the glycine–serine spots when it is desired to separate these amino acids by reversed irrigation in the phenol – aqueous pyrophosphate solvent. An appropriate part of the chromatogram, such as the larger area indicated in broken lines at the top left-hand corner of 1A (in Fig. 1), is removed before the remainder is dipped in the ninhydrin solution. When the color has been developed, the position of glycine–serine spots can be judged (e.g. as indicated by broken circles in 1A) on the detached portion, which is then trimmed down to a suitable size for the reversed chromatography. It is unnecessary to eliminate either methionine-sulphone or hydroxyproline from the bottom of this paper since these do not interfere, being carried well beyond serine or glycine during the upward irrigation by the phenolic solvent.

For the detection of the spots on the small chromatogram, most of the phenol is removed in a draft of air at 40° C; then ethyl acetate is allowed to creep up the paper from the bottom while the paper is held sloping in a shallow dish. After drying, the paper is dipped in the usual ninhydrin solution supplemented by a few milligrams of hydrindantin, e.g. ca. 3 mg in 50 ml.

The Propanol–Ethanol–Pyrophosphate (P-E-PP) System

Specification of Optimum Conditions of Operation of the P-E-PP System

The pattern of separations of the amino acids selected as optimum for the P-E-PP system is shown in Fig. 1 (chromatogram 1A). Chromatogram 1B illustrates the separation of serine and glycine in an excised portion of the standard P-E-PP chromatogram by a second short irrigation in the phenolic – aqueous pyrophosphate solvent (cf. below). While the pattern shown in 1A was selected as affording the most generally useful resolution of amino acids, there may be circumstances, as will be seen later, in which the selection of other forms of the basic P-E-PP pattern would be advantageous. It is proposed first to specify conditions under which the pattern shown in 1A is obtained with high reproducibility in all its essential features. These conditions will be described in the Sections (*a*)–(*d*) below.

(a) The P-E-PP Chromatogram

The chromatograms for the selected P-E-PP system (and for all the variants of it to be illustrated below) consist of the Whatman No. 3 paper impregnated with the specified buffer solution 1 (pyrophosphate). The effective wick length (of Whatman No. 1 paper) will be discussed below in connection with the

adjustment of the flow rate.

(b) The Selected P-E-PP Solvent

The composition and method of preparing the solvent for the selected P-E-PP system have been described.

(c) Preconditioning of the P-E-PP System

For the preconditioning of the selected P-E-PP system, the closed unit, prior to addition of the chromatographic solvent, should contain 9 g water and 4 ml *n*-propanol (ignoring the small amount of water present initially in the air in the empty 'dry' unit and ignoring also any slow exchange of moisture between the polyethylene-wrapped absorption pads and the rest of the system). Accordingly, if the pair of chromatograms contain x g imbibed water when placed in the unit, then $(9-x)$ ml water, followed by 4 ml *n*-propanol, is pipetted into the receiver tube of the closed unit. The motor-driven fan is then turned on for a period of 30 minutes, by which time the added liquid is adequately distributed within the system. The required volume of the P-E-PP solvent (see below) is then added to the trough via a long-stemmed funnel inserted through the small hole in the glass lid of the unit. The commencement of the irrigation may be postponed for periods of 2–3 hours after the cessation of fanning, if desired, but the fan should not be left running for more than 45 minutes owing to slight heating effects. The amount of imbibed water in the chromatograms (x) is estimated either by weighing a reference chromatogram or by deducing it from a reading of the relative humidity of the ambient atmosphere with which the chromatograms are approximately in equilibrium. For the latter method it is convenient to construct a curve from the data given in Table I from which the imbibed water corresponding to any observed value of relative humidity can be read.

(d) The Optimum Rate of Solvent Flow in the P-E-PP System

The optimum rate of solvent flow is defined as the rate which will cause leucine to travel 21–24 inches during 40 hours of irrigation of the standard chromatogram at 34° C. For the particular batches of Whatman No. 3 and No. 1 paper which have been used in much of the work for the chromatograms and feeder wicks, respectively, the desired rate was obtained with a feeder wick 54 mm long and a solvent volume of 225 ml; this corresponds to an effective wick length ca. 42 mm (cf. Part 2, Table I). For other batches of No. 3 and No. 1 papers, effective wick lengths ranging from 33–48 mm have been found to give the desired travel of leucine in the 40-hour period.

It should be noted that an element of operational convenience has entered into the selection of 40 hours as the standard irrigation period. As will be illustrated later, almost equally good patterns of separation can be obtained in slightly shorter periods, such as 30 hours, but such intervals are inconvenient to operate as a routine. Separations which are less perfect but which may be adequate for some purposes can be obtained in still shorter periods, such as 15 to 20 hours. Recent observations have indicated that some textures of paper

are able to tolerate appreciably higher rates of irrigation than others, without deterioration in the resolving capacity.

Influence of Variations in Conditions of Operation of P-E-PP Systems

The extensive observations reported in Part 3 of this series (2) provided the main basis for adapting the P-E-PP system for operation with the type 4 chromatographic unit. With the improved control of the initial state so afforded, some minor alterations proved to be desirable but in general the conditions specified above as yielding the optimum pattern of separations are close to those which would have been predicted from the earlier work with the type 3 chromatographic unit.

(a) Variations in the Composition of P-E-PP Solvents

It was unnecessary to reinvestigate wide variations in solvent composition but solvents of the following rather similar compositions were compared in the type 4 unit, the preconditioning being with 8–9 g water and 4 ml *n*-propanol in each case:

Solvent	*n*-Propanol	Ethanol	Buffer solution 1 (pyrophosphate)
1	40 vol.	40 vol.	20 vol.
2	48	30	22
3	55	22.5	22.5
4	45	30	25
5	50	25	25
6	60	15	25

These solvents all gave fair patterns of separation, yielding 14–16 separated spots from a test mixture of 19 amino acids. However, solvents 1, 2, 3, and 6, in varying degree showed some crowding of the back portion of the pattern with consequent poor resolution of some of the slow-running amino acids. Solvents 4 and 5, and especially the latter, were superior in this respect.

The sodium chloride content of the specified P-E-PP solvent was selected on the basis of a new series of experiments which confirmed the effects reported earlier (2); the positioning of lysine and arginine, in relation to glutamic acid, histidine, and glycine + serine, was found again to be controlled by adjustment of the sodium chloride content of the solvent. The level of 0.31 ± 0.02 g per 100 ml solvent components was judged best for resolving these amino acids.

(b) Variations in the Preconditioning of the P-E-PP System

With the introduction of preconditioning by direct adjustment of the amounts of volatile constituents present in the initial state, it was shown readily that the main requirement was to control the initial water content of the system. However, the addition of 4 or 6 ml *n*-propanol was found to be beneficial in producing slightly rounder spots, particularly in the case of the fast-running amino acids; larger amounts produced no further benefit and a standard addition of 4 ml *n*-propanol was adopted. The addition of ethanol caused some

undesirable effects, including increased size of spot and a less favorable linear distribution of the amino acids as had been observed earlier with the type 3 unit (2). Accordingly the selected preconditioning of the P-E-PP system involves the addition of water and *n*-propanol only.

Variations in the initial water content of the system caused characteristic differences in the chromatograms, as had also been foreshadowed in the observations presented in Part 3 (2). In Fig. 2 (2A–2F) are shown a series of chromatograms preconditioned with increasing amounts of water from 3 to 12 g, the standard volume of 4 ml *n*-propanol being added in all cases.

With increasing initial amounts of water, the distance of travel of all the amino acids during the 40-hour irrigation tends to increase progressively, but with some irregularities, especially in the fast-running group. The increases in movement of the different amino acids are unequal, however, and with increasing water, there results a more uniform distribution of the amino acids over the available length of the chromatogram. The advantages of a moderately high initial water content are evident from the patterns of separation in 2A to 2F, which show an important opening out of the crowded slow-moving sequence of amino acids (aspartic acid to hydroxyproline); the length of chromatogram occupied by the faster-running amino acid is correspondingly decreased but the resolutions are not generally impaired by this. Certain features of this series of patterns deserve specific mention as follows:

(i) The double nature of the aspartic acid – cysteic acid spot (accentuated by the differential ninhydrin-staining of the two, mentioned previously) becomes more evident with increasing water.

(ii) Although the presence of lysine, histidine, and arginine is discernible even with the smallest amount of water (3 g) the separation is improved with increasing amounts of water up to at least 8–10 g.

(iii) Hydroxyproline, freely separated with the lower amounts of water, becomes crowded between methionine-sulphone and threonine when the water is increased to 10–12 g.

(iv) Confirming the conclusions reached in Part 3 of these studies, the three amino acids tryptophan, methionine, and phenylalanine are accelerated slightly more than their neighbors with increasing amounts of initial water; this brings about a characteristic distribution, easily recognizable from the forward portion of the chromatogram, as a transformation of a 'dry' to a 'wet' P-E-PP pattern.

(v) The partial separation of leucine and isoleucine into a 'dumb-bell' shape is evident at all water levels, but it occurs most noticeably and reliably in the range 8–10 ml water. This partial separation permits the separate analysis of these two amino acids by cutting across the 'waist' of the double spot, with an error which will be defined subsequently. [The complete separation of leucine and isoleucine has been obtained so far only under conditions in which other features are impaired cf. Part 2 (2).]

(vi) There is a progressive increase in the size of spot for a given loading as the initial amount of water is increased; with some batches of paper this may

become a serious disadvantage with amounts of initial water greater than 10 g.

Consideration of the above specific features, in conjunction with the accumulated experience of the general quality and reproducibility of the separations, have led to the adoption of the recommended 9 g as the optimum initial amount of water for general analytical purposes; it will be appreciated that this is not a sharply defined optimum and variations in the range 8–10 g water produce only minor differences. Furthermore, the selection of this particular pattern is to some extent arbitrary and for special purposes different preconditioning may well prove to offer advantages. For example, if it were desired especially to separate and determine methionine without subjecting the amino acid mixture to an oxidative treatment (to convert methionine to methionine-sulphone) this could be done reliably by operating with only 3 g initial water in this unit instead of 9 g. Other special requirements encountered from time to time may similarly be met by the *ad hoc* choice of the conditions in the light of the adjustability of the pattern of separations.

A further aspect of the new method of preconditioning deserves comment here. Four examples of chromatograms produced under the recommended conditions of operation for the P-E-PP system are illustrated. The total initial amount of water was 9 g in each case but very different proportions of this total amount were introduced as imbibed water already present in the papers themselves. The examples referred to are chromatograms 1A, 2D, 2G, and 2H; the water imbibed in the pairs of chromatograms in these cases amounted to 2, 4, 8, and 3 g in the order mentioned. The similarity of these patterns of separation in all essential features illustrates the versatility of the new method of preconditioning and suggests that it will prove effective under extremely varied climatic conditions. Certain minor features of the patterns, so far of little consequence, appear to be influenced significantly by whether the papers are very dry or very moist when they are placed in the units. It is probable that full air-conditioning of the laboratory at some moderate level of humidity such as 40% relative humidity would afford control of these minor variations, but, at the present stage, these appear to be less important than other remaining variations, caused in part at least by variability in paper structure.

(c) Variations in Duration and Rate of Solvent Flow in the P-E-PP System

The selected optimum pattern of separations appears to be obtainable most reliably under the specified conditions of irrigation, the rate of flow of solvent being regulated to cause leucine to travel about 23 inches in 40 hours. The remaining chromatograms in Fig. 2, 2H–2L illustrate the effects of variations from these standard conditions of irrigation. In 2H and 2L, for example, the duration of the irrigation has been reduced from 40 hours to 15 hours, respectively, the flow rate and other conditions being the same. Although many of the resolutions are inferior in 2L the basic pattern of separations has been developed in the 15-hour period. In chromatograms 2I and 2J the irrigation periods were reduced to 30 hours and 24 hours, respectively, but the flow rates

were adjusted to produce almost the normal distance of travel of leucine (ca. 20 inches) in these shorter periods. In both cases the resolutions were fairly satisfactory although less perfect in several respects than the standard P-E-PP chromatograms. For example, both show poor separations of the basic amino acids, (lysine and histidine were superimposed in 2I) and in 2J no separation of leucine and isoleucine was visible. It is interesting to compare the patterns of separation in 2J and 2K, these being obtained with the fastest and slowest flow rates in the series, respectively; the flow rate in 2K was so restricted that leucine had travelled only 14 inches in the full standard 40-hour period of irrigation. Despite the limited migration, however, the actual separations in 2K over the whole pattern were superior to those in 2J, in which the amino acids had travelled much further but much more rapidly.

These varied examples serve to illustrate the points made earlier that there is considerable latitude in the flow rates and durations of irrigation which will yield useful patterns of separation and that the recommended 40-hour period is based partly upon the chromatographic advantages and partly upon convenience in operation.

The Propanol–Ethanol–Tartrate (P-E-T) System

Development of a Complementary System

Difficulty is to be anticipated in attempting to develop a chromatographic system for the avowed purpose of separating the solutes left unresolved in another established system (in this case the P-E-PP system); not only are the requirements as to the additional separations inflexible but these are likely to include inherently difficult cases. A brief outline of the exploratory work leading to the selection of the P-E-T system illustrated in Fig. 1 will indicate the nature of the difficulties encountered, including some which remain to be overcome for an ideal solution of the problem.

The search for the complementary system was begun when it seemed that, in the presence of glutamine and asparagine, the following groups of amino acids would remain unresolved in the P-E-PP pattern: aspartic + cysteic acids, lysine + asparagine, histidine + glutamine + arginine, and finally serine + glycine. Reference to Fig. 5A will show the positions of asparagine and glutamine in relation to the other amino acids which form the back portion of the P-E-PP pattern.

A review of patterns of separation observed in early trials with acidic solvents, consisting of miscible alcohols and aqueous buffers, suggested that the desired resolutions might be obtained in such a system since the same groups of nine amino acids formed the back part of the pattern (and hence could be separated from the rest of the amino acids) but, owing to the difference in pH, they occurred in a very different order (cf. 2). The distribution in systems buffered in the range pH 3–4 with tartrate seemed most promising.

Influence of Variations in Conditions of Operation of P-E-T Systems

(a) Preliminary Studies

The selection of the recommended P-E-T system illustrated in chromatogram 1C (Fig. 1), to be specified in detail below, was based upon the study of many variables. These included the use of tartrate buffers ranging in concentration from 0.02–0.20 *M* and in pH from 3.0–4.5, of solvents of widely different composition, and of varied preconditioning treatments. Many of these observations require only brief mention; for example, the buffer solution 2 (0.10 *M* tartrate, pH 3.4) was selected early as giving the most advantageous distribution of the slow-moving amino acids, and this buffer has been used both for impregnation of the papers and for addition to the solvents in all the systems which will be illustrated below. None of the solvents to be mentioned contained chloride. It was found, as in the case of P-E-PP systems, that addition of chloride ion caused differential acceleration of the basic amino acids but, from the nature of the pattern of separations, this effect could not be used to advantage. The remaining topics to be discussed and illustrated relate to the adjustment of the pattern by variations in composition of solvent, flow rate of solvent, and preconditioning.

(b) Variations in the Composition of P-E-T Solvents

An early pattern of separations obtained with a P-E-T system, the solvent containing 50 vol. *n*-propanol – 25 vol. ethanol – 25 vol. aqueous tartrate buffer, was illustrated in Part 3 of this series (system No. 6, Fig. 2). Tests with the type 4 chromatographic unit were made first with this same solvent and then with variants of it such as the solvents used for chromatograms 3A–3G and 4A (Figs. 3 and 4). (In these eight cases the systems were preconditioned with 4 ml *n*-propanol and 6 g total water, and the flow rates were adjusted to cause leucine to travel about 22 inches in 40 hours.)

This selection of chromatograms illustrates well the potentialities and limitations of the tartrate-buffered systems, as regards the separation of the slow-moving sequence of amino acids from lysine to serine. In general the chromatographic characteristics will be seen to be inferior to those of the P-E-PP chromatograms, the spots being usually more diffuse and, in some cases, elongated; these undesirable features are accentuated by the presence of ethanol in the solvent. The effects of decreasing ethanol at two levels of water concentration may be seen by comparing 3A and 3E (with 30 vol.% water) and the series 3B, 4A, 3C, and 3F (with 35 vol.% water). The influence of these variations upon the back portion of the pattern are slight but they were found to be consistent and, as will be seen, of determining importance insofar as the particular separations required of this system are concerned.

The presence of ethanol in concentrations of 10–25 vol.% resulted in all cases in an incipient separation of glycine and serine, visible unmistakably from the difference in the ninhydrin coloration of these two amino acids. But this promising feature was accompanied by consistent detrimental effects of the

ethanol, namely, failure of clean separation of any one of the basic amino acids, and the crowding of asparagine between cysteic and aspartic acids, both impair-

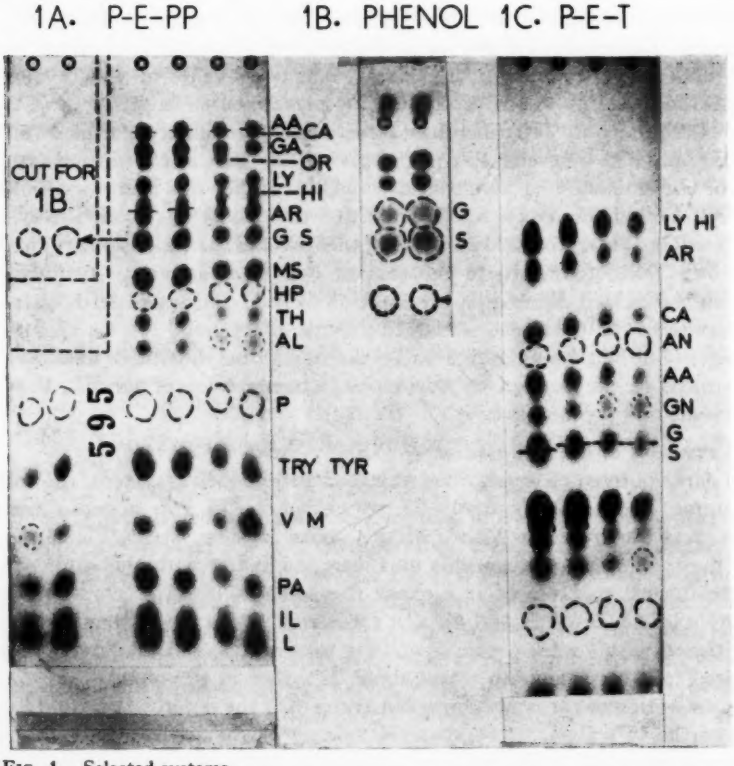

FIG. 1. Selected systems.

Chromatograms 1A and 1C show the finally adjusted patterns of separation of the selected pair of systems. (1A) P-E-PP (*n*-propanol – ethanol – pyrophosphate buffer) system operated as described in the text. Loadings of the six spottings shown (left to right): 0.1, 0.2, 0.3, 0.2, 0.1, 0.1 μmole of each amino acid present, added by single or multiple spotting with ca. 5 μl of marker solution. (1C) P-E-T (*n*-propanol – ethanol – tartrate buffer) system operated as described in the text. Loadings of four spottings shown (left to right): 0.4, 0.3, 0.2, 0.1 μmole of each amino acid present.

Chromatogram 1B shows separation of glycine and serine by reversed chromatography in phenol – pyrophosphate solvent applied to excised portion of chromatogram 1A shown in broken lines. Original position of glycine–serine spot shown by broken circles in 1B. Abbreviations as in footnote.*

*ABBREVIATIONS USED in labelling chromatograms: AA, aspartic acid; αAB, α-aminobutyric acid; βAB, β-aminobutyric acid; αAIB, α-aminoisobutyric acid; βAIB, β-aminoisobutyric acid; AL, alanine; βAL, β-alanine; AN, asparagine; AR, arginine; CA, cysteic acid; CYSTEINE-MAL, cysteine-maleimide; *p*FLUORO-PA, *p*-fluorophenylalanine; G, glycine; GA, glutamic acid; GN, glutamine; GSH, glutathione; GSH-MAL, glutathione-maleimide; HI, histidine; HP, hydroxyproline; IL, isoleucine; L, leucine; LY, lysine; M, methionine; MS, methionine-sulphone; MSO, methionine-sulphoxide; OR, ornithine; P, proline; PA, phenylalanine; S, serine; TYR, tyrosine; TRY, tryptophan; V, valine.

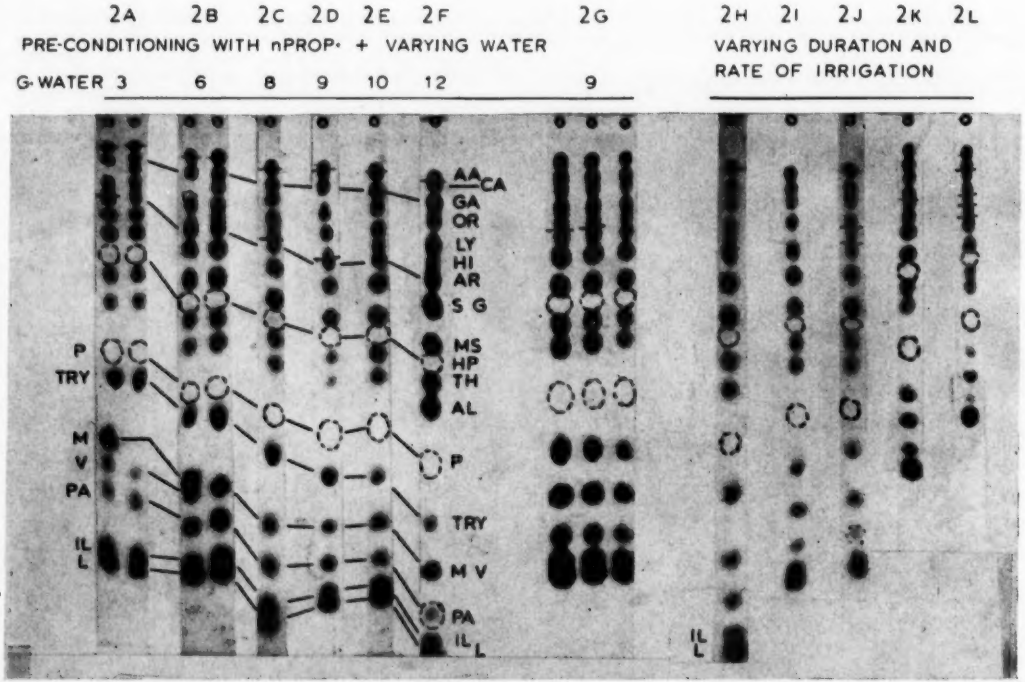

FIG. 2. Influence of varying conditions on P-E-PP system.

Chromatograms 2A–2G show influence of preconditioning with varying total initial amounts of water (with constant 4-ml addition of *n*-propanol). Loadings of each amino acid present: (2A) 0.1, 0.1 μmole; (2B) 0.1, 0.2 μmole; (2C–2F inclusive) 0.2 μmole; (2G) 0.3, 0.2, 0.1 μmole.

Chromatograms 2H–2L show influence of varying the duration of irrigation and flow rate of solvent. The relative flow rates can be judged from the distance of travel of (say) leucine in the following periods of irrigation: (2H) 40 hours, (2I) 30 hours, (2J) 24 hours, (2K) 40 hours, (2L) 15 hours. Loadings of each amino acid present: 0.1 μmole throughout. For abbreviations see footnote to Fig. 1.

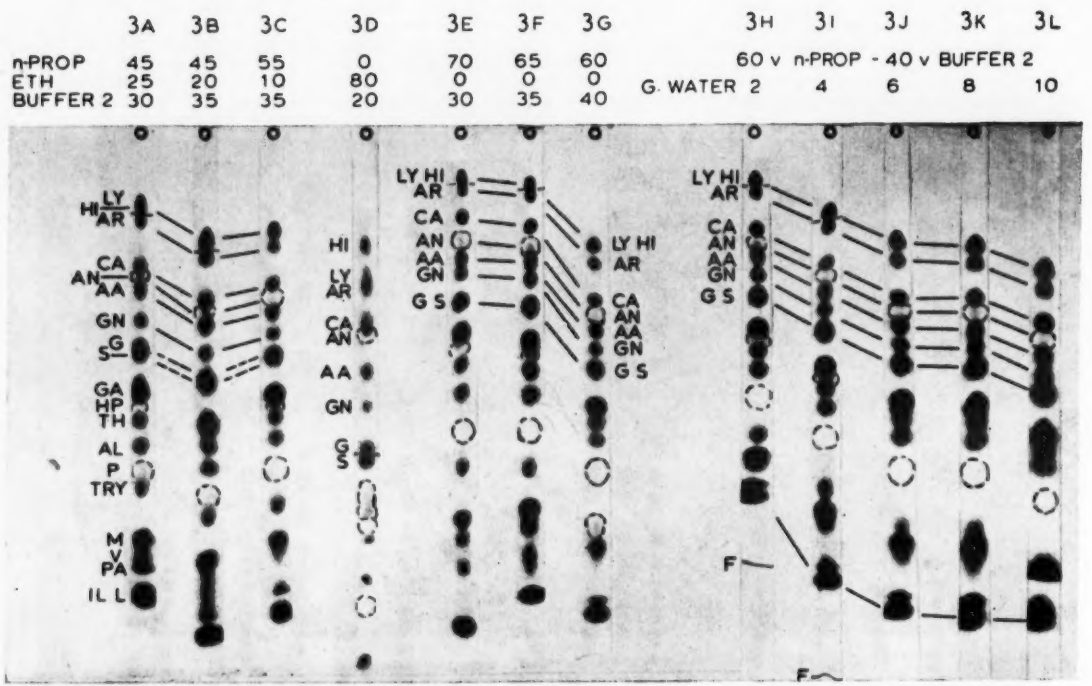
3A 3B 3C 3D 3E 3F 3G 3H 3I 3J 3K 3L
n-PROP 45 45 55 0 70 65 60
ETH 25 20 10 80 0 0 0
BUFFER 2 30 35 35 20 30 35 40
60 v n-PROP - 40 v BUFFER 2
G. WATER 2 4 6 8 10
LY HI AR
CA AN AA GN G S
GA HP TH
AL P TRY
M V PA IL L
F

FIG. 3. Influence of varying conditions in P-E-T systems.

Chromatograms 3A–3G show patterns obtained with range of *n*-propanol, ethanol, tartrate buffer mixtures used with tartrate-buffered paper. (Compositions as shown above.) Loadings: (3A) 0.2; (3B) 0.3; (3C) 0.2; (3D–3G inclusive) 0.1 μmole.

Chromatograms 3H–3L show influence of preconditioning with varying total initial amounts of water (with constant 4-ml addition of *n*-propanol). Loadings of each amino acid present: 0.2 μmole. For abbreviations see footnote to Fig. 1.

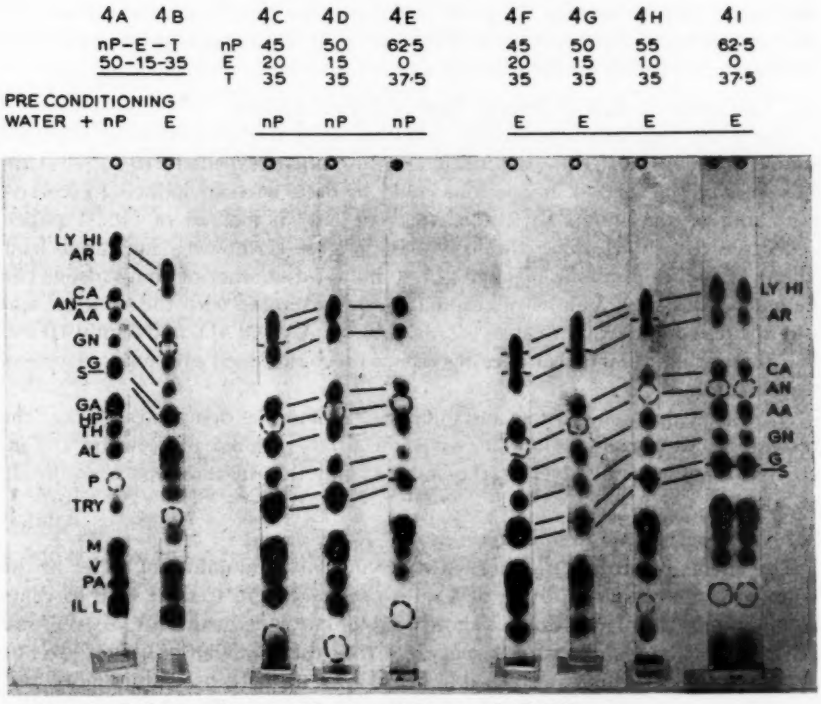
4A 4B 4C 4D 4E 4F 4G 4H 4I
nP-E-T nP 45 50 62·5 45 50 55 62·5
50-15-35 E 20 15 0 20 15 10 0
T 35 35 37·5 35 35 35 37·5
PRE CONDITIONING
WATER + nP E nP nP nP E E E E
LY HI AR
CA AN AA
GN G S
GA HP TH
AL
P
TRY
M V PA IL L
LY HI AR
CA AN AA GN G S

FIG. 4. Influence of preconditioning and flow rate in P-E-T systems.
The influence of addition of 4 ml *n*-propanol as compared with 10 ml ethanol during preconditioning is shown for different solvents. For example 4A, 4C, 4D, and 4E were preconditioned with 4 ml propanol (+ 6 g total water) and all others with 10 ml ethanol (+ 6 g total water). The flow rate for chromatograms 4C–4I was much increased as compared with 4A and 4B, so that fast-running amino acids are run off. Loadings of all amino acids present: 0.2 μmole throughout except in 4I where loadings 0.4 and 0.3 μmole are shown. For abbreviations see footnote to Fig. 1.

ing the required separations. The pattern in chromatogram 3D, containing only ethanol and aqueous tartrate buffer, is interesting in showing almost complete separation of glycine and serine, and a regrouping of the basic amino acids, histidine being the slowest and lysine contiguous with arginine.

It seemed unlikely at first that effective use could be made of the rudimentary separation of glycine and serine obtainable by the inclusion of ethanol in low concentrations, owing to the detrimental effects mentioned above. Accordingly, the influence of varying the water content in *n*-propanol – aqueous tartrate buffer solvents was tested extensively and a few examples are 3E, 3F, and 3G. These tests showed that a level of 37.5–40 vol.% of water, the remainder being *n*-propanol, could be used to obtain reliably most of the required resolutions as seen in 3G, and for a time this particular system was adopted as the

optimum tartrate system. It gave no separations of glycine and serine, and the separation of asparagine was clean only with the lighter loadings of amino acids, e.g. 0.1 or 0.2 μmole at most.

(c) Variations in Rate of Solvent Flow in P-E-T Systems

Since the required separations involved only the slow-running sequence amino acids of the P-E-T pattern, it was an obvious expedient to increase the flow rate of the solvent as much as could be done without inducing excessive streaking of the spots. By using wicks of No. 3, instead of No. 1 paper, (effective length 34 mm) the travel of glycine–serine was increased from about 11 inches to about 17 inches in 40 hours; a number of comparisons can be made from Figs. 3 and 4 of chromatograms irrigated with the same solvent but at these different flow rates: e.g. 3B and 4C, 4A and 4D, and finally 4B and 4G, the last pair being examples of systems preconditioned with ethanol instead *n*-propanol.

A close comparison of these and many other examples demonstrated that the resolutions were consistently improved in all the desired respects by the increased distances of travel of the solutes, and this feature was accordingly of adopted.

(d) Variations in the Preconditioning of P-E-T Systems

The effects of preconditioning with varying initial amounts of water in the unit (with a constant addition of 4 ml *n*-propanol were studied with solvents having the same compositions as those used for chromatograms 3A, 3B, 3C, and 3G and, in all cases, the results suggested that there was an optimum level at about 6 g water. This was studied in detail with the solvent containing 60 vol. *n*-propanol – 40 vol. aqueous tartrate buffer, in the series illustrated in chromatograms 3H–3L. The general effects of increasing the initial water are shown strikingly in these photographs of the medium loading (0.2 μmole); the specific effects on the slow-running amino acids which led to the selection of 6 g water as the optimum are: (1) with 2 g and 4 g there is no clean separation of arginine (or other basic amino acid) and the aspartic acid spot is elongated and trailing; (2) with 8 g and 10 g water the spot size, increasing progressively throughout the series, becomes excessive and results in contiguity of most of the spots. This is more serious the heavier the loading. Accordingly, 3J shows the best separations of the lysine–serine sequence; asparagine is somewhat crowded between cysteic and aspartic acids, appreciably more so in 3J with 0.2-μmole loading than in 3G with 0.1-μmole loading.

The effect of the alcohol added during preconditioning was studied. In view of its mixed beneficial and detrimental effects, ethanol had been eliminated as a component of the irrigating solvent (*cf.* above). Experiments were now carried out to determine whether the beneficial effects alone might be obtained by using ethanol (and water) to replace the *n*-propanol (and water) in preconditioning the tartrate-buffered systems.

Figure 4 illustrates in part this final phase of the adjustment of the P-E-T

system. The effects of preconditioning with 10 ml ethanol, instead of 4 ml *n*-propanol, may be seen for a number of different solvents by referring to the following pairs of chromatograms: 4A and 4B, 4C and 4F, 4D and 4G, 4E and 4I. It should be mentioned that all these chromatograms are of the medium (0.2-μmole) loading except 4A, which was 0.1 μmole, and 4I, which shows both 0.3- and 0.4-μmole loadings. In each case some adverse effects of preconditioning with ethanol may be seen, namely, poorer separation of arginine from lysine + histidine and, in most cases, slightly more diffuse spots; against these features, however, were a more definite separation of glycine and serine (made visible by the different ninhydrin colorations) and a small but important increase in the space between cysteic and aspartic acids, due in part to improved migration characteristics of the latter. It will be seen that the patterns of separations in 4I, even for the heavily loaded spots in this case, show quite clean separations of arginine, cysteic acid, asparagine, aspartic acid, and glutamine, and a partial separation of glycine and serine which is sufficient to demarcate approximately the boundary between them.

This system illustrated in chromatogram 4I and previously in 1C was chosen as the nearest approach to the desired complementary system for the P-E-PP system.

Specification of Optimum Conditions of Operation of the P-E-T System

It has been emphasized sufficiently that the optimum pattern of the P-E-T system has been judged solely on the extent to which its separations supplemented those obtained in the selected P-E-PP system. In the system finally selected the solvent contains no ethanol but it is still referred to as a 'P-E-T' system since preconditioning with ethanol is required to obtain the resolutions which have been illustrated in chromatograms 1C and 4I (Figs. 1 and 4). As in the P-E-PP system, there may be circumstances in which some variant from the selected P-E-T system would offer advantages; for example, the separations obtained by using a slower flow rate so that all amino acids remain on the chromatogram will be referred to below (cf. Fig. 5B).

The conditions for obtaining patterns similar in all essential features to those shown in 1C and 4I will be described in Sections (*a*)–(*d*) below.

(a) The P-E-T Chromatogram

The chromatograms for the P-E-T system consist of the Whatman No. 3 paper impregnated with the specified buffer solution 2 (tartrate). In order to obtain the relatively long migrations of the amino acids, a feeder wick of Whatman No. 3 paper is used. Although this is the same as the chromatogram itself it is found preferable to sew on the wick in the usual way since this facilitates its handling and, especially, its accurate and secure mounting without squeezing of the paper between the support rods (cf. Part 2 (1)). It avoids also the need for purchasing a longer size of sheet for the purpose.

(b) The Selected P-E-T Solvent

The composition and method of preparing the solvent has been described.

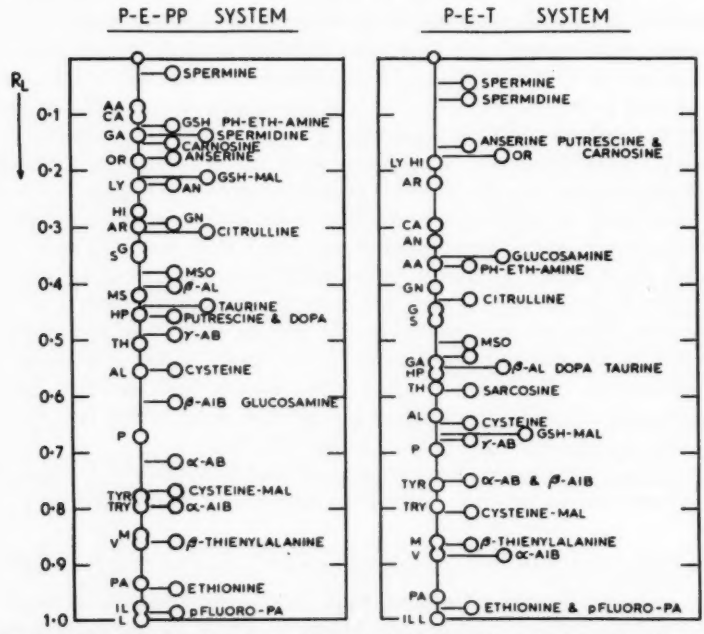

FIG. 5. Distribution of amino acids and derivatives in relation to leucine in the P-E-PP and P-E-T systems.

The conditions were as finally recommended, except that the slower flow rate for the P-E-T system was used so that leucine remained on the chromatogram. For abbreviations see footnote to Fig. 1.

(c) Preconditioning of the P-E-T System

In the initial state prior to addition of the chromatographic solvent, the selected P-E-T system should contain 10 ml ethanol and 6 g water (ignoring moisture present initially in the air of the empty 'dry unit' and any moisture exchange with the polyethylene-wrapped absorption pads). Accordingly, if the pair of chromatograms contains x g imbibed water when placed in the unit, then $(6-x)$ ml water, followed by 10 ml ethanol, is pipetted into the receiver tube of the unit. The procedure thereafter is exactly as described earlier for the P-E-PP system.

In the selected system the fast-running amino acids (valine, phenylalanine, isoleucine, and leucine) run off the chromatogram into the pad, so that the flow rate is defined as that which will cause glycine or serine to travel 14–17 inches during 40 hours of irrigation at 34° C. This rate is not critical but if glycine–serine travel much less than 14 inches the separation of cysteic acid, asparagine, and aspartic acid may be imperfect; on the other hand, if glycine–serine travel much more than 17 inches in the 40 hours, elongation of all spots becomes noticeable due to excessive flow rate.

As was mentioned above, the chromatograms are provided with feeder wicks of Whatman No. 3 paper. The desired flow rate has been obtained with effective wick lengths of between 32 and 44 mm with different batches of paper (cf. Part 2 (1) Table I).

Discussion

The specific purpose of this communication has been to describe the development of a complementary pair of one-dimensional chromatographic systems designed to separate nearly all the common amino acids in the convenient operating period of 40 hours (two nights and one day). These were developed by following a general approach outlined in the preceding communications (1, 2) which consists essentially of attempting to identify successive factors influencing different features of the chromatograms and to adjust each of these, in turn, to obtain the most favorable possible pattern of separations. Accordingly, a wider purpose of the communication has been to draw attention to the probability that useful improvements in the operation of many other paper chromatographic systems can be expected to result from the application of this same approach. These different aspects of the work will be considered briefly.

The development of the two systems referred to as the P-E-PP and P-E-T systems, respectively, has posed distinctive problems in the two cases. The final P-E-PP system is the result of a concerted attempt to obtain maximum resolution of the whole group of 20 or so amino acids within the available length of about 24 inches of filter paper in the physical system adopted. Once the most promising combination of paper type, buffer salts, and solvent components had been selected, the problem resolved itself into choosing at each stage conditions yielding the most advantageous linear spacing, coupled with desirable spot characteristics (compactness, freedom from distortion, etc.) over the whole length of the chromatogram; it was frequently necessary to forego the possibility of securing some particular desired feature because it could be obtained only in conjunction with some impairment of the chromatography in another region of the pattern. In contrast to this requirement for optimum over-all performance in the P-E-PP system, the requirement of the P-E-T system was limited but more specific; in order to form an effective complement for the first system it was necessary to secure additional resolutions amongst the nine amino acids (including asparagine and glutamine) forming the back portion of the P-E-PP pattern. The choice of the tartrate-buffered system was governed by this consideration from the outset even though it had been recognized that acidic buffered systems tended to show generally inferior chromatographic properties as compared with neutral or alkaline systems. Furthermore, in developing the P-E-T system, adverse effects upon any but the back group of amino acids could be ignored and, in fact, under the recommended final conditions the forward part of the pattern is allowed to run into the absorption pad.

Viewed as an analytical tool for the range of amino acids present in protein,

including asparagine and glutamine, the selected complementary pair of systems, with or without the auxiliary system for glycine and serine illustrated in Fig. 1, promises to be useful in many different connections, especially in constitutional studies. Despite the high reproducibility of both systems in nearly all important respects, there remain weaknesses and limitations which will be made clear. If tryptophan, glutamine, and asparagine can be assumed to be absent (e.g. after prolonged acid hydrolysis of a protein and if methionine and cysteine are oxidized to methionine-sulphone and cysteic acid, then these and all the other amino acids likely to be encountered, including ornithine, can be determined. If tryptophan and the two amides are present, then some additional procedure is required for the full analysis. One of these, to be described in Part 7 of this series, involves the separation by a simple treatment on a miniature column of sulphonated polystyrene resin of the group, lysine, histidine, arginine, and tryptophan, from the other amino acids. This is followed by the separate chromatography of the two groups in the P-E-PP system and the usual chromatography of the whole in the P-E-T system. This results in separated individual spots of all the amino acids. If, alternatively, tryptophan and tyrosine are to be determined by measurements of ultraviolet light absorption, and if glutamine and asparagine are present, the determinations of two important amino acids (lysine and histidine) are indirect and rest upon differences.* Other weaknesses in the chromatography should be mentioned. The partial separations of isoleucine and leucine in the P-E-PP system, and more particularly of glycine and serine in the P-E-T system, while reproducible, are capable of yielding only approximate estimates of the proportions of these pairs of amino acids. Finally there remains a certain variability in the positioning of histidine in relation to arginine in the P-E-PP system so that a clean separation of these two cannot be relied upon. Fortunately the separation of arginine in the P-E-T system is dependable.

The two systems have been used to examine a number of cell and tissue extracts and various body fluids in which a great variety of additional amino compounds may be encountered. In Fig. 5 are shown the distributions, in the two systems, of some 25 amino compounds additional to the amino acids found in proteins. (In the P-E-T system here illustrated the flow rate was adjusted to cause leucine to travel 22–24 inches in the 40-hour period.) It should be mentioned that the general level of chromatographic quality in the series of chromatograms on which Fig. 5 has been based was good, the spot characteristics of nearly all these compounds being nearly ideal in the P-E-PP system; even glutathione and cysteine showed only moderate trailing, oxidation presumably being inhibited by the pyrophosphate.

*For example, using the abbreviations indicated in footnote to Fig. 1, with subscripts P and T to denote the P-E-PP and P-E-T systems, respectively, the values for LY and HI are derived as follows:

(1) $LY = (LY+AN)_P - AN_T$,
(2) $HI = (LY+HI)_T - LY$ from (1),
(3) $HI = (HI+GN+AR)_P - GN_T - AR_T$.

It should be mentioned also that the P-E-PP system has been used successfully to provide one separational axis in two different two-dimensional procedures. In one of these, the finished P-E-PP chromatogram of the standard size was irrigated laterally in the form of a cylinder by ascending chromatography with the phenol – aqueous pyrophosphate solvent described earlier. The positions of 38 out of 42 of the compounds shown in Fig. 5, which had been applied as a mixture in a single spot, were discernible on the two-dimensional chromatogram. The development of other procedures particularly for the separation of peptides from complex mixtures is in progress.

In conclusion, some wider implications of the results which have been presented require brief comment. These suggest that improved effectiveness of other chromatographic systems is likely to result from adjustment and closer control of the operating conditions and that a fuller exploitation of the paper chromatogram as a more refined and dependable tool requires equipment designed consciously to permit the easy variation and control of a variety of factors which are obviously capable of influencing the pattern of separations obtained. Finally, the possibility of major refinements of the paper chromatogram which is suggested by the high separational efficiency of systems based on buffered papers and buffered solvents—recognized by McFarren (9) and extended in the present studies—emphasizes the need for fuller elucidation of the separatory mechanism of the functioning chromatogram. Progress on these more fundamental questions, which have been discussed in outline in Part 1 of this series (1), may be expected to extend still further the refinement of this remarkable tool, by providing the basis for rational and integrated systems of design and control, extending from the details of paper structure to the maintenance of selected gradients of the separatory field on the chromatogram and including many features of which no more than semiempirical use can be made at present.

Acknowledgments

We wish to thank Miss D. D. Wood for technical assistance and for invaluable help in the difficult task of recording and collating the intangible information involved in this work. We wish to acknowledge the generous support of this investigation by grants from the National Research Council of Canada and the National Cancer Institute of Canada. Two of the authors (C.K.H. and M.A.M.) held Fellowships from the National Cancer Institute of Canada whilst participating in this investigation.

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APPENDIX TO PART 4

NOTE ON MOISTURE RELATIONS OF CHROMATOGRAPHIC PAPERS

C. S. HANES AND D. D. WOOD

The hygroscopic properties of cellulosic materials, including papers of many types, have been studied in different connections, and various implications in relation to paper chromatography have been pointed out (1, 2, 3). In considering above the preconditioning of chromatographic systems, it was mentioned that the amount of imbibed water in the filter papers is a variable which is neither controlled adequately nor taken into account in normal chromatographic procedures. When the importance of this variable became clear the studies to be summarized below were undertaken. Their object was to define the relationship between the ambient atmospheric humidity and the moisture content of the chromatograms of Whatman No. 3 papers of the standard size, both in the unbuffered state and after impregnation with different buffer solutions as they were prepared for use in various chromatographic systems.

Experimental

The moisture relations of the chromatograms were studied in two different experiments, as follows:

(1). Two or three chromatograms of each of eight selected types were exposed to a sequence of controlled humidities in a small 'constant climate' room, the relative humidity being held for a time at each of the following levels: 21, 56, 65, 76, 82, 75, 66, 56, 32, 17, 46, 63, and 74%, the temperature being 21–23° C. Then the temperature was raised to 34° C (at which temperature the chromatography is conducted) and the relative humidity was adjusted in turn to 52, 62, and 20%. Under each set of conditions, each paper was weighed until the weight was constant (to within 40 mg), a minimum period of 12 hours being allowed.

(2). In the second type of experiment, which extended over a period of 18 months, the changes in moisture content were followed under the conditions of uncontrolled humidity in the laboratory. Chromatograms of four different types were kept freely exposed to the atmosphere of the laboratory, being suspended in a screened-in cupboard). During periods of 2–3 weeks at different seasons of the year, the papers were weighed once or twice daily and, at the same times, determinations of the ambient relative humidity were made by sling psychrometer or later by hair hygrometer, when the reliability of the latter was established.

In both experiments, initial estimates of the dry matter of each paper were made (from determinations on control sheets) and direct determinations were made finally (by 3 hours' heating at 120° C in open-ended cylindrical wraps of aluminum foil).

Results

In the first experiment, at controlled humidities, the vapor pressure isotherms showed the typical 'hysteresis loop' reflecting differences in the moisture content at a given relative humidity depending on whether the measurement was made after an increase or a decrease in humidity. This may be seen in Fig. 6, which shows for three types of chromatograms, the individual points observed during uptake and loss limbs of the cycles. For each type a median curve is drawn in Fig. 6, which may be assumed to represent approximately the true equilibrium

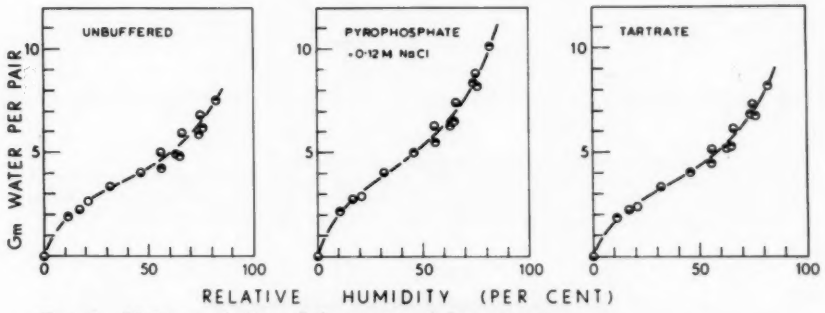

FIG. 6. Moisture relations of three types of chromatograms.
The graphs show the water content per pair of chromatograms of standard size (29½ × 11 in.) of Whatman No. 3 paper, unbuffered and impregnated with pyrophosphate and tartrate buffers as specified for the P-E-PP and P-E-T systems. Initial points, 0; after increase in relative humidity, ◓; after decrease in relative humidity, ◒.

relation. Similar median curves were derived for the eight types of chromatogram included in the experiments. From these, the values shown in Table II have been derived. The results are expressed in terms of grams of water per 100 grams dry filter paper; with the batches of Whatman No. 3 paper used mainly in this investigation the average dry weight of the standard size of chromatogram was 35.5 g so that the moisture contents of the pair of chromatograms introduced into a chromatographic unit would be 71% of these values (cf. Table I).

It will be seen from the data in Table II that the various buffered salt solutions with which these chromatograms had been impregnated resulted in considerable modification in the moisture-holding properties.

The observations made at 34° C, although not extensive, show that the moisture content / relative humidity relation is not significantly altered by a rise in temperature from 22° to 34° C. Accordingly the data in Table II may be used, if desired, to deduce approximately the distribution of water between

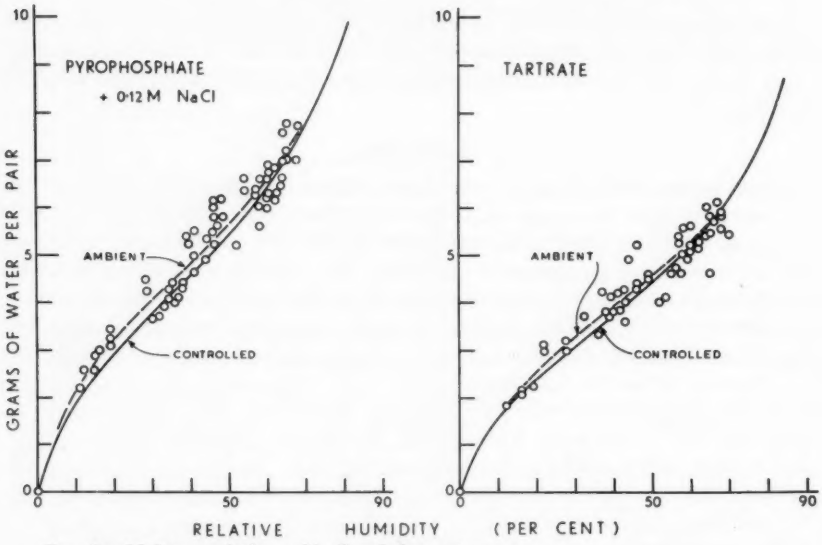

FIG. 7. Moisture relations of buffered chromatograms.
The individual points (circles) represent weighings during uncontrolled fluctuations in the ambient humidity of the laboratory, and the broken line an estimated mean curve. The continuous curve is derived from the observations at controlled humidities (Table II).

TABLE I

Moisture relations of selected chromatograms

	Grams water per two chromatograms of standard size*								
Relative humidity	10	20	30	40	50	60	70	80	85
Type of chromatogram									
Unbuffered No. 3	1.54	2.66	3.50	4.12	4.62	5.28	6.16	7.66	8.94
Pyrophosphate buffered (for selected P-E-PP system)	1.88	2.95	3.72	4.52	5.40	6.30	7.66	9.70	11.40
Tartrate buffered (for selected P-E-T system)	1.73	2.54	3.16	3.80	4.48	5.28	6.24	7.84	9.20

*The average dry weight per pair of chromatograms (29½×11 in.) of the particular batches of Whatman No. 3 paper was 71 g. The moisture contents have been calculated on this basis from the values given in Table II for types 1, 3, and 6.

the papers and the vapor phase in the chromatographic units at the operating temperature of 34° C.

As was to be expected, the observations made in the second experiment, with the fluctuating ambient humidity of the laboratory, showed appreciable scatter. This may be seen in Fig. 7, in which are plotted the points for only two types of chromatogram, namely those buffered as specified for the selected P-E-PP and P-E-T systems, respectively. In each case is included the median line for the corresponding type of chromatogram as determined in the first experiment

TABLE II

Moisture relations of unbuffered and variously buffered chromatograms

Chromatogram type*	1	2	3	4	5	6	7	8
Buffer	None	*M*/20 PP pH 8.9	*M*/20 PP pH 8.9	*M*/20 PP pH 8.9	*M*/20 PP pH 7.1	*M*/10 tart. pH 3.4	*M*/20 tart. pH 3.4	*M*/20 tart. pH 3.4
Including chloride to give:	—	0.05 *M* NaCl	0.12 *M* NaCl	0.24 *M* NaCl	0.16 *M* NaCl	None	0.16 *M* NaCl	0.16 *M* KCl
Relative humidity, %	Grams water per 100 grams dry filter paper (salt free)†							
10	2.16	2.44	2.65	2.80	2.66	2.42	2.38	2.44
20	3.74	4.19	4.16	4.42	4.17	3.56	3.84	4.10
30	4.92	5.44	5.24	5.66	5.21	4.45	5.18	5.30
40	5.80	6.36	6.37	6.88	6.34	5.38	6.31	6.20
50	6.51	7.24	7.61	8.17	7.49	6.31	7.14	7.02
60	7.43	8.45	8.88	9.90	8.78	7.14	8.70	8.11
70	8.67	10.4	10.8	12.4	10.8	8.8	10.7	10.1
80	10.8	11.9	13.7	16.5	14.2	11.1	13.8	13.1
85	(12.6)	(13.6)	(16.1)	(19.3)	(16.6)	(13.0)	(16.0)	(15.9)

*The various types here included have been in use in chromatographic systems for separating different classes of compound. Types 3 and 6 are, respectively, the buffered chromatograms used in the selected P-E-PP and P-E-T systems, types 5, 7, and 8 have been used in systems for separating nucleotides to be described later. (The abbreviations PP and tart. represent pyrophosphate and tartrate respectively.)

†The observations were made on Whatman No. 3 paper, batches K-4883-5, without purification, impregnated in the manner described in Part 2. Two chromatograms of the standard size of this paper weighed 71 g (average).

(controlled). This deviates only slightly from the curve judged to represent the average values obtained in the second experiment (ambient); the slightly higher values in the latter experiment probably reflect the fact that the weighings were made during the day, when the humidity of the laboratory was usually decreasing.

The results indicate that it is possible to estimate the moisture content of a pair of chromatograms to within approximately 1 g of water either by the weighing of reference chromatograms, or by making a determination of the atmospheric humidity and reading the moisture content from the median curve constructed from the data presented in Table II. This degree of accuracy appears to be adequate for controlling the preconditioning of the various chromatographic systems so far tested.

Acknowledgment

The main experiment described in this note was carried out at the Ontario Research Foundation, Toronto, in a chamber provided with controlled humidity and temperature. We are grateful to Dr. A. E. R. Westman for permission to use this chamber and to Dr. S. G. Reid and Mr. N. Morrison, who generously assisted in its operation.

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